

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
16 January 2003 (16.01.2003)

PCT

(10) International Publication Number  
WO 03/004644 A1

- (51) International Patent Classification<sup>7</sup>: C12N 15/11 (74) Agent: FB RICE & CO; 139 Rathdowne Street, Carlton, Victoria 3053 (AU).
- (21) International Application Number: PCT/AU02/00897 (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.
- (22) International Filing Date: 5 July 2002 (05.07.2002) (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- (25) Filing Language: English (71) Applicant (for all designated States except US): COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, Australian Capital Territory 2601 (AU).
- (26) Publication Language: English
- (30) Priority Data: PR 6215 6 July 2001 (06.07.2001) AU
- (72) Inventors; and (75) Inventors/Applicants (for US only): WHYARD, Steven [CA/AU]; 125 Fullagar Crescent, Higgins, Australian Capital Territory 2615 (AU). CAMERON, Fiona, Helen [AU/AU]; 28 Chelmsford Avenue, Lindfield, New South Wales 2070 (AU). MOGHADDAM, Minoo [AU/AU]; 39 Elva Avenue, Killara, New South Wales 2071 (AU). LOCKETT, Trevor, J. [AU/AU]; 38 Terry Road, Denistone, New South Wales 2114 (AU).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/004644 A1

(54) Title: DELIVERY OF dsRNA TO ARTHROPODS

(57) **Abstract:** The invention is to methods of gene silencing in arthropods using dsRNA. The method is to include contacting the arthropod with, and/or directly feeding the arthropod, the dsRNA to the arthropods to deliver the dsRNA to arthropod tissues. It is envisaged that the methods of the invention will have use in determining the biological function of genes in arthropods. Methods of pest control of arthropods, and of protecting arthropods against parasites and predators are provided. Transgenic arthropods expressing dsRNA molecules are also provided by the present invention.

## Delivery of dsRNA to Arthropods

### Field of the Invention

The present invention relates generally to dsRNA and its use in gene silencing. Furthermore, the present invention relates to methods of delivering dsRNA to an arthropod.

### Background of the Invention

RNA interference (RNAi) is considered as a naturally occurring adaptive defence in at least some organisms against viruses and the production of aberrant transcripts, such as those produced by transposon mobility (Bosher and Labouesse, 2000; Waterhouse *et al.*, 2001).

The actual process by which dsRNA mediates target RNA degradation is not fully understood, but the cellular machinery involved is gradually being identified. Full-length dsRNAs have been observed to be progressively degraded into ~21-nucleotide dsRNAs, by an enzyme called Dicer-1 (Elbashir *et al.*, 2001). It is believed that the Dicer-1 proteins, along with their associated 21-mer dsRNA, seek single stranded RNAs with sequence identity, and promote the cleavage of single stranded RNA targets (Waterhouse *et al.*, 2001).

The intestine of *C. elegans* is a simple tube constructed of only 20 cells (White, 1988). For *C. elegans*, dsRNA has been microinjected into the gonadal tissues of adult worms, but simpler methods have since been developed that circumvent the tedious microinjection method. Nematodes fed *Escherichia coli* bacteria that simultaneously express sense and antisense RNAs can acquire dsRNA. Interestingly, the ingested dsRNA can then spread from the gut to target almost all tissues with the nematode (Timmons and Fire, 1998). Alternatively, the worms can be soaked in dsRNA solutions, either with liposomes or as naked RNA (Tabara *et al.*, 1998; Maeda *et al.*, 2001).

Arthropod guts are comprised of a great many cell types, and are highly variable, as they have adapted to the needs of each species and their unique dietary choices. The evolutionary distance between nematodes and insects is considerable, and there is no reason to assume that while feeding dsRNA to *C. elegans* was successful, it would be a technique easily transferable to insects. The presence of specific barriers in insect guts, such as the peritrophic membrane, could also limit or prevent direct absorption of orally delivered

dsRNA. The midgut of an arthropod is the primary site of nutrient uptake, and midgut internal environments of different arthropods can vary widely. For example, the fruit fly *Drosophila melanogaster* has a rather acidic midgut lumen, while many Lepidoptera (moths and butterflies) have a very hostile, 5 highly basic midgut environment.

### Summary of the Invention

The present invention provides methods that utilize dsRNA to determine the biological function of an RNA in an arthropod. In particular, the invention 10 provides efficient mechanisms of delivering dsRNA to an arthropod with the aid of transfection promoting agents. Furthermore, the present invention provides methods for controlling pest arthropod populations, methods for controlling pathogens carried by arthropods, as well as methods for protecting an arthropod from a pathogen, parasite or predatory organism. In addition, the 15 present invention provides transgenic organisms, in particular arthropods, expressing small dsRNA molecules.

In one aspect, the present invention provides a method of determining the biological function of a target RNA in an arthropod comprising delivering to the arthropod a dsRNA molecule which specifically reduces the level of the 20 target RNA and/or the production of a protein encoded by the target RNA in a cell of the arthropod, and assessing the effect of the dsRNA on at least one biological function of the arthropod.

The method of the present invention can be utilized to rapidly screen uncharacterized RNAs or expressed sequence tags (ESTs) for a function(s), 25 particularly in high(er) throughput screens of pest arthropod EST libraries. Ultimately, the method facilitates the identification of novel pesticide targets. For example, a particular dsRNA that confers lethality on an arthropod indicates that the corresponding RNA itself, or the protein encoded by a mRNA, 30 is essential for arthropod survival, and, as a consequence, said RNA or protein is a good pesticide target. Accordingly, this RNA, or the protein encoded by the mRNA, is specifically targeted in the design of, and/or screening for, agents to control pest populations of the arthropod.

In an alternative embodiment, the method is used to determine any additional function(s) of previously characterized arthropod RNAs.

35 Alternatively, dsRNA is designed with specificity to an RNA that is potentially involved in particular biological processes (for example, determined

by sequence identity with known genes, and/or through expression patterns) and can be screened to obtain a dsRNA that produces a particular phenotype. Such phenotypes include arthropod death or sterility. In fact, random dsRNA can be screened by this method for a desired phenotype.

- 5 Pathogens, such as viruses, which infect arthropods can be engineered to express a dsRNA for the down-regulation of a specific RNA. Typically, this would be for the production of biological agents to control a pest population of arthropods. However, such pathogens may not be easily manipulated, slowing down the progress of identifying suitable genetically engineered pathogens.
- 10 The present invention can be used to rapidly screen candidate dsRNA molecules to determine if they produce the desired effect on a target arthropod pest. Once a candidate has been shown to produce the desired effect, suitable pathogens can be engineered and tested as biological control agents of an arthropod population.
- 15 The method of the invention can also be used to identify RNA important for enhancing production traits of an arthropod. In this instance, the activity of the dsRNA can down-regulate the production trait. Once identified, the relevant genes can be overexpressed to enhance these production traits. In accordance with this embodiment of the invention, the corresponding
- 20 endogenous arthropod gene is ectopically expressed in the arthropod to enhance the production trait. Exemplary production traits contemplated herein include the composition and/or quantity of honey produced by bees, and the growth rate and/or size of edible crustaceans such as prawns, crayfish and lobsters, and the like.
- 25 In an alternate use of the method of the present invention, a target RNA can be assessed to determine whether it, or a protein encoded by the RNA, is acted upon by an agent such as a pesticide. In this instance, the method also comprises exposing the arthropod to the agent, wherein if the agent has little or no additional effect on the arthropod it indicates that the RNA, or protein
- 30 encoded by the RNA, is directly acted upon by the agent or is involved in a biological pathway which is effected by the agent. Upon the identification of the mechanism of action of the agent, this information can be used to design alternate pesticides (for example) which act on the same molecules/pathways. This is particularly useful where an agent is known to be a potent pesticide,
- 35 however, it is not approved for use due to concerns such as its toxicity to non-pest organisms.

In a preferred embodiment, the dsRNA is delivered by a process comprising contacting the arthropod with the dsRNA. Preferably, said contacting comprises wholly or partially soaking the arthropod in a composition comprising the dsRNA.

- 5 In a further preferred embodiment, the dsRNA is delivered by a process comprising feeding the dsRNA to the arthropod.

Preferably, the dsRNA is delivered in a composition comprising a transfection promoting agent. More preferably, the transfection promoting agent is a lipid-containing compound.

- 10 In one embodiment, the lipid-containing compound is selected from the group consisting of; Lipofectamine, Cellfectin, DMRIE-C, DOTAP and Lipofectin. In another embodiment, the lipid-containing compound is a Tris cationic lipid. Examples of suitable Tris cationic lipids include, but are not limited to, CS096, CS102, CS129, CS078, CS051, CS027, CS041, CS042, 15 CS060, CS039, or CS015.

- Preferably, the composition further comprises a nucleic acid condensing agent. The nucleic acid condensing agent can be any such compound known in the art. Examples of nucleic acid condensing agents include, but are not limited to, spermidine (N-[3-aminopropyl]-1,4-butanediamine), protamine 20 sulphate, poly-lysine as well as other positively charged peptides. Preferably, the nucleic acid condensing agent is spermidine or protamine sulfate.

In yet another preferred embodiment, the composition further comprises buffered sucrose or phosphate buffered saline.

- In an alternate embodiment, the dsRNA is delivered by a process 25 comprising feeding a transgenic organism expressing the dsRNA to the arthropod. The transgenic organism is selected from, but not limited to, the group consisting of: plants, yeast, fungi, algae, bacteria or another arthropod expressing the dsRNA. Examples of suitable bacteria include *Pseudomonas fluorescens*, *E. coli*, *B. subtilis* (Gawron-Burke and Baum, 1991), and 30 *Wolbachia* sp. Preferably, the transgenic organism is a transgenic plant.

In yet another embodiment, the dsRNA is delivered by a process comprising contacting the arthropod with a virus expressing the dsRNA.

- Preferably, the dsRNA comprises a nucleotide sequence having at least 90% identity to at least a portion of the sequence of the target RNA, more 35 preferably the dsRNA comprises a nucleotide sequence having at least 97% identity to at least a portion of the sequence of the target RNA, and even more

preferably the dsRNA comprises a nucleotide sequence having at least 99% identity to at least a portion of the sequence of the target RNA.

The dsRNA has a region of self-complementarity to permit it assuming a double-stranded conformation in an arthropod host. Preferably, the region of 5 self-complementary corresponds to at least about 20 to about 23 contiguous nucleotides of the target RNA, more preferably the full length sequence of the target RNA.

The arthropod can be any species. Preferably, the arthropod is of economic importance, such as, for example, an edible crustacean, an 10 arthropod that causes disease, a household pest, an agricultural pest, or an arthropod that produces a useful substance or compound, such as, for example, silk, an edible substance (e.g. honey) or a medicinal substance or compound (e.g. a toxin or venom).

It is preferred that the arthropod is an insect or a crustacean. Most 15 preferably the arthropod is an insect.

The arthropod can be at any stage of development, however, it is preferred that the arthropod is in a larval or adult developmental stage when the dsRNA is delivered. The present invention clearly encompasses determining the effect of the dsRNA on a phenotype of the arthropod at a later 20 development stage even when the dsRNA is delivered at an earlier developmental stage.

Preferably, the RNA is mRNA.

In a further embodiment, the dsRNA molecule is designed based on the nucleotide sequence of an EST that has been derived from mRNA isolated 25 from the arthropod.

In another aspect, the present invention provides a composition comprising dsRNA and a transfection promoting agent, wherein said dsRNA comprises a nucleotide sequence that it is at least 90% identical to the sequence of a target RNA, wherein the target RNA is selected from the group 30 consisting of: a naturally-occurring arthropod RNA, a naturally-occurring RNA of an organism that is a pathogen carried by an arthropod, a naturally-occurring RNA of a virus that infects an arthropod, an RNA copy of a naturally-occurring DNA virus that infects an arthropod, and a naturally-occurring RNA of a bacterium that infects an arthropod.

It is preferred that the naturally occurring arthropod RNA is an mRNA which encodes a protein involved in, and more preferably essential for, arthropod development, neural function, reproduction or digestion.

Preferably, the transfection promoting agent is a lipid-containing compound.

In one embodiment, the lipid-containing compound is selected from the group consisting of; Lipofectamine, Cellfectin, DMRIE-C, DOTAP and Lipofectin. In another embodiment, the lipid-containing compound is a Tris cationic lipid. Examples of suitable Tris cationic lipids include, but are not limited to, CS096, CS102, CS129, CS078, CS051, CS027, CS041, CS042, CS060, CS039, or CS015.

Preferably, the composition further comprises a nucleic acid condensing agent. The nucleic acid condensing agent can be any such compound known in the art. Examples include, but are not limited to, spermidine (N-[3-aminopropyl]-1,4-butanediamine), protamine sulphate, poly-lysine as well as other positively charged peptides. Preferably, the nucleic acid condensing agent is spermidine or protamine sulfate.

Preferably, the composition is formulated such that it can be applied to an area inhabited by a population of arthropods. This area can include crop plants, ornamental or native plants, or animals. Furthermore, the composition can be applied directly to an animal such as a cow or a sheep. Accordingly, in a preferred embodiment the composition further comprises an agriculturally acceptable carrier.

The composition of the present invention can also be formulated as a bait. In this instance, the composition further comprises a food substance and/or an attractant, such as a pheromone, to enhance the attractiveness of the bait to the arthropod.

In a further aspect, the present invention provides a method of controlling an arthropod pest comprising delivering to the arthropod dsRNA by a process comprising contacting the arthropod with said dsRNA or feeding said dsRNA to the arthropod, for a time and under conditions sufficient for said dsRNA, or a degradation product thereof, to specifically reduce the level of a target RNA and/or the production of a protein encoded by the target RNA in a cell of the arthropod, wherein the target RNA or the protein is important for arthropod survival, development and/or reproduction.

Preferably, the dsRNA is delivered in a composition according to the invention.

Preferably, the target RNA or the target protein is essential for arthropod development, neural function, reproduction or digestion.

5 The present invention is also used to control disease pathogens carried by arthropods. For instance, there are ecological arguments for not destroying mosquitoes to control malaria, sleeping sickness, and many arboviruses.

Accordingly, in yet another aspect, the present invention provides a method for controlling a pathogen transmitted by an arthropod, the method 10 comprising delivering to the arthropod dsRNA by a process comprising contacting the arthropod with said dsRNA or feeding said dsRNA to said arthropod, for a time and under conditions sufficient for said dsRNA, or a degradation product thereof, to specifically reduce the level of a target RNA and/or the production of a protein encoded by the target RNA in a cell of the 15 pathogen, wherein the target RNA or the protein is important for pathogen survival, development and/or reproduction.

Preferably, the dsRNA is delivered in a composition according to the invention.

In preferred embodiment of the third aspect, the pathogen is selected 20 from the group consisting of fungi, protozoans, bacteria and viruses.

In the instance where the pathogen is a virus, the presence of the dsRNA, or degradation products thereof, in a cell of the arthropod specifically reduces the accumulation of a target RNA or the production of a protein essential for viral survival and/or replication.

25 Beneficial arthropods can be protected from parasite/pathogen attack by the delivery of appropriate dsRNA containing compositions. Insect colonies, in particular those such as bees, silkworms, or even laboratory stocks of insects, can be protected from parasitic or predatory pests (eg. nematodes, mites), or viral and microbial pathogens. Similarly, commercially important stocks of 30 crustaceans can be protected from disease pathogens.

Thus, in a further aspect the present invention provides a method of protecting an arthropod against a pathogen, parasite or predatory organism, the method comprising delivering to the arthropod dsRNA by a process comprising contacting the arthropod with said dsRNA or feeding said dsRNA to 35 said arthropod, for a time and under conditions sufficient for said dsRNA, or a degradation product thereof, to specifically reduce the level of a target RNA

and/or the production of a protein encoded by the target RNA in a cell of the pathogen, parasite or predatory organism, wherein the target RNA or the protein is important for the survival, development and/or reproduction of the pathogen, parasite or predatory organism.

- 5 Preferably, the dsRNA is delivered in a composition according to the invention.

In the instance where the pathogen is a virus, the presence of the dsRNA, or degradation products thereof, in a cell of the arthropod specifically reduces the accumulation of RNA or the production of a protein essential for 10 viral survival and/or replication.

Previously, dsRNA techniques have involved the use of constructs in which the dsRNA approximates the length of the entire open reading frame of a RNA or a substantial portion thereof. The present inventors have found that such long dsRNA constructs are not required in order to obtain RNA 15 interference. Surprisingly, the present inventors have found that dsRNA as little as 21 nucleotides are capable of gene silencing. Furthermore, the present inventors have also surprisingly found that dsRNA that had been previously processed and partially degraded within one organism can still facilitate RNAi in another arthropod.

20 Hence, in another aspect the present invention provides a transgenic organism comprising a heterologous nucleic acid(s) which is transcribed to produce a dsRNA, wherein the portion of the dsRNA which is double stranded is about 21 to about 50 base pairs in length.

Preferably, the dsRNA comprises a nucleotide sequence having at least 25 90% identity to at least a portion of the sequence of a target RNA selected from the group consisting of: a naturally-occurring arthropod RNA, a naturally-occurring RNA of an organism that is a pathogen carried by an arthropod, a naturally-occurring RNA of a virus that infects an arthropod, an RNA copy of a naturally-occurring DNA virus that infects an arthropod, and a naturally- 30 occurring RNA of a bacterium that infects an arthropod.

Preferably, the portion of the dsRNA which is double stranded is about 21 to about 23 base pairs in length.

Preferably, the organism is selected from the group consisting of: plants and arthropods.

In the instance where the transgenic organism is a plant, the dsRNA is preferably at least 90% identical to at least a portion of a RNA expressed in an arthropod which feeds on the plant.

Preferably, the dsRNA increases the resistance of the transgenic 5 organism to a pathogen. Preferably, the pathogen is a virus.

Preferably, the dsRNA is produced as a single open reading frame in the transgenic organism, where the sense and anti-sense sequences are flanked by an unrelated sequence which enables the sense and anti-sense sequences to hybridize to form the dsRNA molecule with the unrelated sequence forming a 10 loop structure.

As will be apparent, preferred features and characteristics of one aspect of the invention can be applicable to many other aspects of the invention.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a 15 stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of 20 providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

The invention will hereinafter be described by way of the following non- 25 limiting Figures and Examples.

#### **Brief Description of the Drawings**

Figure 1. PCR detection of phspGUS[i/r] plasmid in insects injected with the plasmid as late embryos. In the leftmost 7 lanes, the presence of the GUS 30 transgene in the flies is evident by the production of the 1 kb PCR product in all developmental stages. There was no PCR product in wild type, non-transgenic flies (wt). In the right hand side of the gel, only L1 (1<sup>st</sup> instar larvae) show evidence of the injected plasmid, as indicated by the single 500 bp PCR product.

Figure 2. Gene silencing in *D. melanogaster* larvae and adults after feeding neonates dsRNA. Neonate larvae were soaked in a composition comprising transfection promoting agent and GUS dsRNA, and individuals were assayed either as 2<sup>nd</sup> instar larvae (top panel) or as adults (bottom panel). A total of 40  
5 individuals were assayed for each group. Each dot represents one individual's level of GUS gene silencing, relative to non-treated controls

Figure 3. Reduced GUS activity following soaking of neonate larvae in a composition comprising transfection promoting agent and different  
10 concentrations of dsRNA. Each dot represents one individual adult fly's GUS activity, as a percentage of non-treated GUS controls. A total of 20 flies were assayed for each concentration of dsRNA.

Figure 4. Effectiveness of different transfection promoting agents on the oral  
15 delivery of dsRNA to neonate *D. melanogaster* larvae. A total of 20 larvae were soaked in different transfection promoting agents containing 1 ug/ul dsRNA, and the GUS activity was assessed in 2<sup>nd</sup> instar larvae.

Figure 5. Gene silencing in *D. melanogaster* larvae after feeding neonates  
20 dsRNA, without the presence of spermidine in the RNA mixture. Neonate larvae were soaked in a composition comprising transfection promoting agent and GUS dsRNA, and individuals were assayed as 2<sup>nd</sup> instar larvae. A total of 25 individuals were assayed, with each dot representing one individual's level of GUS gene silencing, relative to non-treated controls.

25 Figure 6. GUS gene silencing in *D. melanogaster* fed RNA extracts derived from *D. melanogaster* adults that had been injected with the dsRNA expression plasmid phspGUS[i/r] as embryos. The top panel illustrates the range of gene silencing in 3<sup>rd</sup> instar larvae previously fed the extracted RNA, and the bottom  
30 panel shows the range of gene silencing observed in adult flies. Each dot represents a single individual insect. A total of 20 individuals were assayed for each group.

**Detailed Description of the Invention****General Techniques**

- Unless otherwise indicated, the recombinant DNA techniques utilized in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, *A Practical Guide to Molecular Cloning*, John Wiley and Sons (1984), J. Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), *Essential Molecular Biology: A Practical Approach*, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), *DNA Cloning: A Practical Approach*, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (Editors), *Current Protocols in Molecular Biology*, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present) and are incorporated herein by reference.
- Standard methods for the production of transgenic insects are outlined in "Insect Transgenesis -- Methods and Applications" (Ed. A.M. Handler and A.A. James, CRC Press, London, 2000).

**dsRNA**

- As used herein, "dsRNA" or "RNAi" refers to a polyribonucleotide structure formed either by a single self-complementary RNA strand or at least by two complementary RNA strands. The degree of complementary, in other words the % identity, need not necessarily be 100%. Rather, it must be sufficient to allow the formation of a double-stranded structure under the conditions employed.

Preferably, the % identity of a polyribonucleotide is determined by GAP (Needleman and Wunsch, 1970) analysis (GCG program) using the default settings, wherein the query sequence is at least about 21 to about 23 nucleotides in length, and the GAP analysis aligns the two sequences over a region of at least about 21 nucleotides. In another embodiment, the query sequence is at least 150 nucleotides in length, and the GAP analysis aligns the two sequences over a region of at least 150 nucleotides. In a further embodiment, the query sequence is at least 300 nucleotides in length and the GAP analysis aligns the two sequences over a region of at least 300 nucleotides. In yet another embodiment, the query sequence corresponds to

the full length of the target RNA and the GAP analysis aligns the two sequences over the full length of the target RNA.

The design and production of suitable dsRNA molecules for the present invention is well within the capacity of a person skilled in the art, particularly 5 considering Dougherty and Parks (1995), Waterhouse et al. (1998), Elbashir et al. (2001), WO 99/32619, WO 99/53050 and WO 99/49029.

Conveniently, the dsRNA can be produced from a single open reading frame in a recombinant host cell, wherein the sense and anti-sense sequences are flanked by an unrelated sequence which enables the sense and anti-sense 10 sequences to hybridize to form the dsRNA molecule with the unrelated sequence forming a loop structure.

The two strands can also be expressed separately as two transcripts, one encoding the sense strand and one encoding the antisense strand.

RNA duplex formation can be initiated either inside or outside the cell. 15 The dsRNA can be partially or fully double-stranded. The RNA can be enzymatically or chemically synthesized, either *in vitro* or *in vivo*.

The dsRNA need not be full length relative to either the primary transcription product or fully processed RNA. Generally, higher identity can be used to compensate for the use of a shorter sequence. Furthermore, the 20 dsRNA can comprise single stranded regions as well, e.g., the dsRNA can be partially or fully double stranded. The double stranded region of the dsRNA can have a length of at least about 21 to about 23 base pairs, optionally a sequence of about 21 to about 50 base pairs, optionally a sequence of about 50 to about 100 base pairs, optionally a sequence of about 100 to about 200 25 base pairs, optionally a sequence of about 200 to about 500, and optionally a sequence of about 500 to about 1000 or more base pairs, up to molecule that is double stranded for its full length, corresponding in size to a full length target RNA molecule.

The dsRNA can contain known nucleotide analogs or modified backbone 30 residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiralmethyl phosphonates and 2-O-methyl ribonucleotides.

As used herein, the term "specifically reduce the level of a target RNA 35 and/or the production of a target protein encoded by the RNA", and variations thereof, refers to the sequence of a portion of one strand of the dsRNA being

sufficiently identical to the target RNA such that the presence of the dsRNA in a cell reduces the steady state level and/or the production of said RNA. In many instances, the target RNA will be mRNA, and the presence of the dsRNA in a cell producing the mRNA will result in a reduction in the production of said 5 protein. Preferably, this accumulation or production is reduced at least 10%, more preferably at least 50%, even more preferably at least 75%, yet even more preferably at least 95% and most preferably 100%, when compared to a wild-type cell.

The consequences of inhibition can be confirmed by examination of the 10 outward properties of the cell or organism or by biochemical techniques such as, but not limited to, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), and other immunoassays.

15

#### Transfection Promoting Agent

Transfection promoting agents used to facilitate the uptake of nucleic acids into a living cell are well known within the art. Reagents enhancing transfection include chemical families of the types; polycations, dendrimers, 20 DEAE Dextran, block copolymers and cationic lipids. Preferably, the transfection-promoting agent is a lipid-containing compound (or formulation), providing a positively charged hydrophilic region and a fatty acyl hydrophobic region enabling self-assembly in aqueous solution into vesicles generally known as micelles or liposomes, as well as lipopolyamines.

25 The formulation of polynucleotides encapsulated in lipid-containing compounds is known in the art and described in, for example, "Liposomes: from physical structure to therapeutic applications" (Ed. C.G. Knight. Elsevier Press, 1981).

As used herein;

30 1) CellFECTIN refers to a 1:1.5 (M/M) liposome formulation of the cationic lipid N, N<sup>I</sup>, N<sup>II</sup>, N<sup>III</sup>-tetramethyl-N<sup>I</sup>, N<sup>II</sup>, N<sup>III</sup>-tetrapalmitoylspermine (TM-TPS) and dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water;  
2) Lipofectin refers to a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and 35 dioleoyl phosphatidylethanolamine (DOPE);

- 3) Lipofectamine refers to a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(spermine- carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water;
- 5 4) DMRIE-C refers to a 1:1 (M/M) liposome formulation of the cationic lipid DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide) and cholesterol in membrane-filtered water;
- 5) DOTAP refers to cationic lipid N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate;
- 10 6) CS096: K3C10TChol (a T-shape trilysine head group with a C10 aliphatic spacer attached to a cholesterol hydrophobic domain via Tris molecule);
- 7) CS102: K3C10TL3 (T-shape trilysine with a C10 aliphatic spacer attached to three aliphatic fatty acid (C12) via Tris molecule);
- 8) CS129: K3C7TS3 (T-shape trilysine with a C7 aliphatic spacer attached to
- 15 three aliphatic fatty acid (C18) via Tris molecule);
- 9) CS078: K2C10TL3 (dilysine with a C10 aliphatic spacer attached to three aliphatic fatty acid (C12) via Tris molecule);
- 10) CS051: K3GTL3 (tri-Lysine with a shorter Glycine spacer to three aliphatic fatty acid (C12) via Tris molecule);
- 20 11) CS027: KATP3 (monolysine with a short alanine spacer to three aliphatic fatty acid (C16) via Tris molecule);
- 12) CS041: K3ATL2 (trilysine with a short alanine spacer to two aliphatic fatty acid (C16) via Tris molecule);
- 13) CS042: K3ATL3 (trilysine with a short alanine spacer to three aliphatic fatty
- 25 acid (C16) via Tris molecule);
- 14) CS060: K3C6TL3 (trilysine with a C6 aliphatic spacer to three aliphatic fatty acid (C16) via Tris molecule);
- 15) CS039: K3ATM3 (trilysine with a short alanine spacer to three aliphatic fatty acid (C16) via Tris molecule);
- 30 16) CS015: K3ATP3 (trilysine with a short alanine spacer to three aliphatic fatty acid (C16) via Tris molecule).
- CS096, CS102, CS129, CS078, CS051, CS027, CS041, CS042, CS060, CS039 and CS015 are specific examples of transfection promoting agents suitable for the methods and compositions of the invention, the method for
- 35 synthesizing which is detailed in WO 96/05218, US 5,583,198, US 5,869,606 and US 5,854,224 (see below) and Cameron et al. (1999).

As used in the present invention, the terms "micelle" and "liposome" mean vesicles composed of amphiphilic lipids self-assembled in aqueous solution to form tertiary structures.

Liposomes are unilamellar or multilamellar vesicles of bilayers which

5 have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion may be organised to contain the composition to be delivered.

Cationic liposomes carry positive charges on their hydrophilic head-group forming liposomes that interact with the negatively charged nucleic acid

10 molecules to form a complex. The positively charged liposome/nucleic acid complex binds to the negatively charged cell surface and is internalized predominantly through the endosomal pathway. A proportion of the endosomes, will rupture, releasing their contents of liposome/nucleic acid complex into the cell cytoplasm.

15 Liposomes that are pH-sensitive or negatively charged, entrap nucleic acid rather than complex with it. Since both the nucleic acid and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, nucleic acid can be entrapped within the aqueous interior of these liposomes.

20 One major type of liposomal composition includes phospholipids other than naturally derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic

25 fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

30 Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion

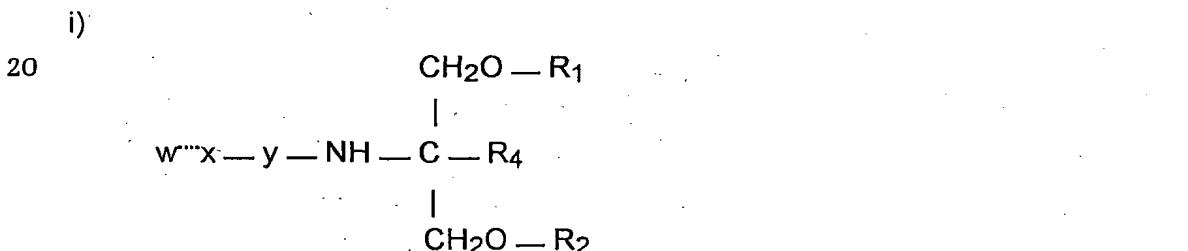
35 of the liposome (A) comprises one or more glycolipids, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety.

- While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of
- 5 the reticuloendothelial system (RES) (Allen and Chonn, 1987; Wu et al., 1993).

A number of liposomes comprising nucleic acids are known in the art. WO 96/40062 discloses methods for encapsulating high molecular weight nucleic acids in liposomes. US 5,264,221 discloses protein-bonded liposomes and asserts that the contents of such liposomes can include an antisense RNA.

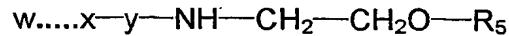
10 US 5,665,710 describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

Transfection promoting agents useful for the methods and compositions of the present invention include "Tris cationic lipids" which are disclosed in WO 15 96/05218, US 5,854,224, US 5,583,198 and 5,869,606, the contents of which are incorporated by reference. These agents include compounds having a formula selected from the group consisting of:



- w is a dsRNA or a nucleic acid encoding a dsRNA
- x is a peptide, amino acid, non-amino acid nucleic acid binding group or non-peptide nucleic acid binding group
- 30 y is a linker having a chain length equivalent to 1 to 20 carbon atoms or is absent
- R<sub>4</sub> is H or CH<sub>2</sub>O - R<sub>3</sub>; and R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are the same or different and are either hydrogen, methyl, ethyl, hydroxyl or an acyl group derived from a fatty acid having a carbon chain of 3 to 24 carbon atoms saturated or
- 35 unsaturated, with the proviso that at least one of R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> is an acyl group derived from a fatty acid;

ii)



5 in which:

w is a dsRNA or a nucleic acid encoding a dsRNA

x is a peptide, amino acid, non-amino acid nucleic acid binding group or non-peptide nucleic acid binding group

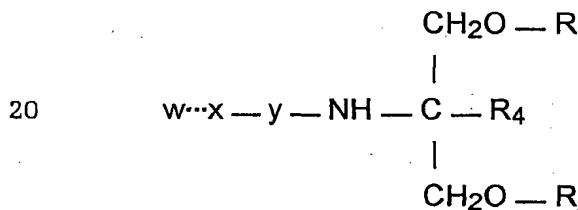
y is a linker having a chain length equivalent to 1 to 20 carbon atoms or

10 is absent

R<sub>5</sub> is an acyl group derived from a fatty acid having a carbon chain of 3 to 24 carbon atoms saturated or unsaturated;

iii)

15



in which:

25 w is a dsRNA or a nucleic acid encoding a dsRNA

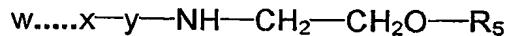
x is a peptide, amino acid, non-amino acid nucleic acid binding group or non-peptide nucleic acid binding group

y is a linker having a chain length equivalent to 1 to 20 carbon atoms or is absent

30 R<sub>4</sub> is H or CH<sub>2</sub>O - R<sub>3</sub>; and R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are the same or different and are either hydrogen, methyl, ethyl, hydroxyl or an acyl group derived from a fatty acid having a carbon chain of 3 to 24 carbon atoms saturated or unsaturated, with the proviso that at least one of R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> is an acyl group derived from a fatty acid;

35

iv)



5 in which:

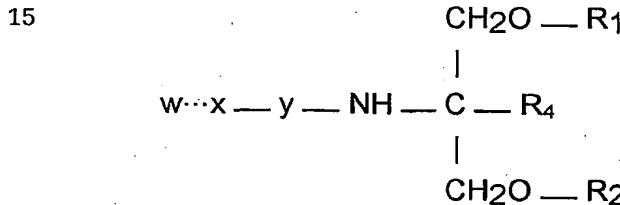
w is a dsRNA or a nucleic acid encoding a dsRNA

x is a peptide, amino acid, non-amino acid nucleic acid binding group or non-peptide nucleic acid binding group

y is a linker having a chain length equivalent to 1 to 20 carbon atoms or 10 is absent

R<sub>5</sub> is an acyl group derived from a fatty acid having a carbon chain of 3 to 24 carbon atoms saturated or unsaturated; and

v)



20 in which:

w is a dsRNA or a nucleic acid encoding a dsRNA

x is a peptide, amino acid, non-amino acid nucleic acid binding group or non-peptide nucleic acid binding group

y is a spacer having a chain length equivalent to 1-30 carbon-carbon 25 single covalent bonds or is absent

R<sub>4</sub> is H or halogen or CH<sub>2</sub>O-R<sub>3</sub>; and R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> are the same or different and are either hydrogen, methyl, ethyl, alkyl, alkenyl, hydroxylated alkyl, hydroxylated alkenyl groups or ether containing alkyl, alkenyl, hydroxylated alkyl or hydroxylated alkenyl groups, optionally being an acyl 30 group derived from a fatty acid having a carbon chain length equivalent to 3-24 carbon atoms saturated or unsaturated, with the proviso that at least one of R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> includes a group having a carbon chain of 3-24 carbon atoms saturated or unsaturated.

Within the meaning of the present invention, the term lipopolyamine 35 denotes any amphiphilic molecule comprising at least one hydrophilic polyamine region and one lipophilic region. The cationically charged polyamine

region of the lipopolyamines is capable of combining reversibly with the negatively charged nucleic acid. This interaction strongly compacts the nucleic acid. The lipophilic region makes this ionic interaction less sensitive to the external medium, by covering the nucleolipid particle formed with a lipid layer.

- 5 Examples of suitable lipopolyamines include those disclosed in US 6,172,048 and US 6,171,612.

Advantageously, the polyamine region of the lipopolyamines used in the context of the invention corresponds to the general formula:



in which m is an integer greater than or equal to 2 and n is an integer greater than or equal to 1, it being possible for m to vary between the different carbon groups included between two amines. Preferably, m is between 2 and 6  
15 inclusive and n is between 1 and 5 inclusive. Still more preferably, the polyamine region is represented by spermine or an analogue of spermine that has retained its properties of binding to nucleic acids.

The lipophilic region can be a saturated or unsaturated hydrocarbon chain, cholesterol, a natural lipid or a synthetic lipid capable of forming lamellar, 20 cubic, or hexagonal phases.

There was some variation in effectiveness of the transfection reagents tested in the arthropod species that were examined. However, considering the present disclosure, it is well within the capacity of the skilled addressee to design routine experiments to test a number of transfection promoting agents to 25 determine which provides the best results for any given arthropod species.

#### Agriculturally Acceptable Carriers

Agriculturally suitable and/or environmentally acceptable compositions for arthropod control are known in the art. Agricultural compositions for the 30 control of arthropod pests of plants and/or animals are preferably suitable for agricultural use and dispersal in fields. Preferably, compositions for the control of other arthropod pests should be environmentally acceptable.

Agriculturally acceptable carriers are also referred to herein as an "excipient". An excipient can be any material that the animal, plant or 35 environment to be treated can tolerate. Furthermore, the excipient must be such that the composition of the present invention is still capable of causing

- gene silencing. Examples of such excipients include water, saline, Ringer's solution, dextrose or other sugar solutions, Hank's solution, and other aqueous physiologically balanced salt solutions, phosphate buffer, bicarbonate buffer and Tris buffer. In addition, the composition may include compounds that
- 5 increase the half-life of a composition. Such compounds are be known to the skilled person in the art.

Compositions of the invention may also comprise agents selected from; conventional pesticides, gustatory stimulants, thickening agents, UV screening agents, optical brighteners, dispersants, flow agents, spreading agents and

10 sticking agents. Preferably, the composition is formulated such that is persist in the environment for a length of time suitable to allow it to be ingested by a target arthropod or contact the target arthropod.

### Arthropods

15 The arthropod can be any organism classified in this taxonomical group. Preferably, the arthropod is selected from the group consisting of: Crustacea, Insecta and Arachnida.

Examples of preferred Insecta include, but are not limited to, members of the orders Coleoptera (e.g. Anobium, Ceutorhynchus, Rhynchophorus, Cospopolites, Lissorhoptrus, Meligethes, Hypothenemus, Hylesinus, Acalymma, Lema, Psylliodes, Leptinotarsa, Gonocephalum, Agriotes, Dermolepida, Heteronychus, Phaedon, Tribolium, Sitophilus, Diabrotica, Anthonomus or Anthrenus spp.), Lepidoptera (e.g. Ephestia, Mamestra, Earias, Pectinophora, Ostrinia, Trichoplusia, Pieris, Laphygma, Agrotis, Amathes, Wiseana, Tryporyza, Diatraea, Sporganothis, Cydia, Archips, Plutella, Chilo, Heliothis, Helicoverpa (especially Helicoverpa armigera), Spodoptera or Tineola spp.), Diptera (e.g. Musca, Aedes, Anopheles, Culex, Glossina, Simulium, Stomoxys, Haematobia, Tabanus, Hydrotaea, Lucilia, Chrysomia, Callitroga, Dermatobia, Gasterophilus, Hypoderma, Hylemyia, Atherigona, Chlorops, Phytomyza, Ceratitis, Liriomyza, and Melophagus spp.), Phthiraptera, Hemiptera (e.g. Aphis, Bemisia, Phorodon, Aeneoplamia, Empoasca, Parkinsiella, Pyrilla, Aonidiella, Coccus, Pseudococcus, Helopeltis, Lygus, Dysdercus, Oxycarenus, Nezara, Aleurodes, Triatoma, Rhodnius, Psylla, Myzus, Megoura, Phylloxera, Adelyes, Niloparvata, Nephrotettix or Cimex spp.), Orthoptera (e.g. Locusta, Gryllus, Schistocerca or Acheta spp.), Dictyoptera (e.g. Blattella, Periplaneta or Blatta spp.), Hymenoptera (e.g.

Athalia, Cephus, Atta, Lasius, Solenopsis or Monomorium spp.), Isoptera (e.g. Odontotermes and Reticulitermes spp.), Siphonaptera (e.g. Ctenocephalides or Pulex spp.), Thysanura (e.g. Lepisma spp.), Dermaptera (e.g. Forficula spp.) and Psocoptera (e.g. Peripsocus spp.) and Thysanoptera (e.g. Thrips tabaci).

- 5 In one embodiment, the Arthropod is not a *Drosophila* sp.

Examples of preferred Arachnida include, but are not limited to, ticks, e.g. members of the genera *Boophilus*, *Ornithodoros*, *Rhipicephalus*, *Amblyomma*, *Hyalomma*, *Ixodes*, *Haemaphysalis*, *Dermocentor* and *Anocentor*, and mites and manges such as *Acarus*, *Tetranychus*, *Psoroptes*, *Notoednes*,

- 10 Sarcoptes, *Psorergates*, *Chorioptes*, *Demodex*, *Panonychus*, *Bryobia* and *Eriophyes* spp.

Examples of preferred Crustaceans include, but are not limited to, crayfish, prawns, shrimps, lobsters and crabs.

15 Recombinant Vectors

Polynucleotides encoding dsRNA useful for the methods and/or compositions of the present invention can be inserted into a recombinant vector. The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and typically is a virus or a plasmid.

- 20 One type of recombinant vector comprises a polynucleotide encoding a dsRNA operatively linked to an expression vector. Alternatively, the two strands of the dsRNA are encoded by separate open reading frames. The phrase operatively linked refers to insertion of a polynucleotide molecule into an expression vector in a manner such that the molecule is able to be

- 25 expressed when transformed into a host cell. As used herein, an expression vector is a DNA or RNA vector that is capable of transforming a host cell and of effecting expression of a specified polynucleotide molecule(s). Preferably, the expression vector is also capable of replicating within the host cell. Expression vectors can be either prokaryotic or eukaryotic, and are typically viruses or

- 30 plasmids. Expression vectors of the present invention include any vectors that function (i.e., direct gene expression) in recombinant cells of the present invention, including in bacterial, fungal, endoparasite, arthropod, other animal, and plant cells. Preferred expression vectors of the present invention can direct gene expression in arthropod cells.

- 35 In particular, expression vectors of the present invention contain regulatory sequences such as transcription control sequences, origins of

replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression of the polynucleotide encoding a dsRNA or a strand thereof. In particular, recombinant molecules of the present invention include transcription control sequences. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in at least one of the recombinant cells of the present invention. A variety of such transcription control sequences are known to those skilled in the art. Preferred transcription control sequences include those which function in arthropod cells. Additional suitable transcription control sequences include tissue-specific promoters and enhancers.

A particularly preferred expression vector is a baculovirus. By "baculovirus" it is meant any virus of the family Baculoviridae, such as a nuclear polyhedrosis virus (NPV). Baculoviruses are a large group of evolutionarily related viruses, which infect only arthropods; indeed, some baculoviruses only infect insects that are pests of commercially important agricultural and forestry crops, while others are known that specifically infect other insect pests. Because baculoviruses infect only arthropods, they pose little or no risk to humans, plants, or the environment.

Of the suitable DNA viruses, in addition to the Baculoviridae are the entomopox viruses (EPV), such as *Melolontha melonotha* EPV, *Amsacta moorei* EPV, *Locusta migratoria* EPV, *Melanoplus sanguinipes* EPV, *Schistocerca gregaria* EPV, *Aedes aogymi* EPV, and *Chironomus liridus* EPV. Other suitable DNA viruses are granulosis viruses (GV). Suitable RNA viruses include togaviruses, flaviviruses, picornaviruses, cytoplasmic polyhedrosis viruses (CPV), and the like. The subfamily of double stranded DNA viruses Eubaculovirinae includes two genera, NPVs and GVs, which are particularly useful for biological control because they produce occlusion bodies in their life cycle. Examples of GVs include *Cydia pomonella* GV (codling moth GV), *Pieris brassicae* GV, *Trichoplusia ni* GV, *Artogeia rapae* GV, and *Plodia interpunctella* GV (Indian meal moth).

Suitable baculoviruses for practicing this invention may be occluded or non-occluded. The nuclear polyhedrosis viruses ("NPV") are one baculovirus

sub-group, which are "occluded." That is, a characteristic feature of the NPV group is that many virions are embedded in a crystalline protein matrix referred to as an "occlusion body." Examples of NPVs include *Lymantria dispar* NPV (gypsy moth NPV), *Autographa californica* MNPV, *Anagrapha falcifera* NPV 5 (celery looper NPV), *Spodoptera littoralis* NPV, *Spodoptera frugiperda* NPV, *Heliothis armigera* NPV, *Mamestra brassicae* NPV, *Choristoneura fumiferana* NPV, *Trichoplusia ni* NPV, *Helicoverpa zea* NPV, and *Rachiplusia ou* NPV. For field use occluded viruses often are preferable due to their greater stability 10 since the viral polyhedrin coat provides protection for the enclosed infectious nucleocapsids.

Among illustrative, useful baculoviruses in practicing this invention are those isolated from *Anagrapha falcifera*, *Anticarsia gemmatalis*, *Buzura suppressaria*, *Cydia pomonella*, *Helicoverpa zea*, *Heliothis armigera*, *Manestia brassicae*, *Plutella xylostella*, *Spodoptera exigua*, *Spodoptera littoralis*, and 15 *Spodoptera litura*. A particularly useful "NPV" baculovirus for practicing this invention is AcNPV, which is a nuclear polyhedrosis virus from *Autographa californica*. *Autographa californica* is of particular interest because various major pest species within the genera *Spodoptera*, *Trichoplusia*, and *Heliothis* are susceptible to this virus.

20

#### Transgenic Plants

The term "plant" refers to whole plants, plant organs (e.g. leaves, stems 25 roots, etc), seeds, plant cells and the like. Plants contemplated for use in the practice of the present invention include both monocotyledonous and dicotyledonous plants. Exemplary dicotyledonous plants include cotton, oilseeds and other brassicas, tomato, tobacco, potato, bean, and soybean. Exemplary monocotyledonous plants include wheat, maize, barley, rice, and sorghum. The choice of the plant species is determined by the intended use of 30 the plant or parts thereof and the amenability of the plant species to transformation.

Transgenic plants, as defined in the context of the present invention include plants (as well as parts and cells of said plants) and their progeny which have been genetically modified using recombinant DNA techniques to produce at least one dsRNA useful for the methods of the present invention in 35 the desired plant or plant organ.

A polynucleotide encoding a dsRNA, or two different polynucleotides encoding individual strands of a dsRNA, may be expressed constitutively in the transgenic plants during all stages of development. Depending on the use of the plant or plant organs, the dsRNA may be produced in a stage-specific 5 manner. Furthermore, depending on the use, the polynucleotides may be expressed tissue-specifically or induced under specific environmental condition such as for example, wounding by an arthropod pest.

Regulatory sequences which are known or are found to cause expression of a polynucleotide(s) encoding a dsRNA of interest in plants may 10 be used in the present invention. The choice of the regulatory sequences used depends on the target crop and/or target organ of interest and the desired mode of expression (e.g. constitutive induced or tissue specific). Such regulatory sequences may be obtained from plants or plant viruses, or may be chemically synthesized. Such regulatory sequences are well known to those 15 skilled in the art.

Other regulatory sequences such as terminator sequences and polyadenylation signals include any such sequence functioning as such in plants, the choice of which are known to the skilled addressee. An example of such sequences is the 3' flanking region of the nopaline synthase (nos) gene of 20 *Agrobacterium tumefaciens*.

Several techniques are available for the introduction of an expression construct containing a polynucleotide(s) encoding a dsRNA of interest into the target plants. Such techniques include but are not limited to transformation of protoplasts using the calcium/polyethylene glycol method, electroporation and 25 microinjection or (coated) particle bombardment. In addition to these so-called direct DNA transformation methods, transformation systems involving vectors are widely available, such as viral and bacterial vectors (e.g. from the genus *Agrobacterium*). After selection and/or screening, the protoplasts, cells or plant parts that have been transformed can be regenerated into whole plants, using 30 methods known in the art. The choice of the transformation and/or regeneration techniques is not critical for this invention.

**Examples****Methods*****GUS RNA in vitro transcription plasmids***

Standard gene cloning methods (Sambrook *et al.*, 1989) were used to 5 make the gene constructs. The GUS gene encoding the bacterial enzyme  $\beta$ -glucuronidase was amplified by PCR from the pBacPAK8-GUS plasmid (Clonetech) using the primers EcoGusF (GAATTCATGGTCCGTCCTGTAGAAACC) (SEQ ID NO: 1) and EcoGusR (GAATTCCCCCACCAGGGCTGTAGC) (SEQ ID NO: 2). The 1.87 kb PCR 10 product was subcloned into the plasmid pGEM3Zf(+) into the EcoR I site using the EcoR I linkers on the primers, creating two plasmids: pGEM3Z-GUS[s] (sense orientation of GUS gene, relative to the T7 promoter); and pGEM3Z-GUS[a/s] (antisense orientation of GUS gene, relative to the T7 promoter). Both plasmids were digested with the restriction endonuclease EcoRV, followed by 15 religation of the plasmid, to remove 213 bp of the GUS ORF. This ensured that no functional GUS enzyme would be produced if the sense GUS RNA was translated. The resultant plasmids, named pGEM3Z- $\Delta$ GUS[s] and pGEM3Z- $\Delta$ GUS[a/s], were used for *in vitro* transcription of sense and antisense GUS RNAs.

20

***GUS RNA in vivo expression constructs***

*In vivo* expression of sense, antisense, and inverted repeat RNA in *D. melanogaster* embryos was achieved by preparing three plasmids that expressed RNA under the control of the *D. melanogaster* heat shock promoter 25 hsp70. A 1 kb fragment containing the hsp70 promoter, a small multiple cloning site, and the heat shock polyadenylation signal was amplified using PCR from the plasmid pCaSpeR-hs (Thummel *et al.*, 1988) using the primers hsp70F (GAATTCTAGAATCCCAAAACAAACTGG) (SEQ ID NO: 3) and hst70R (GGATCCTGACCGTCCATCGCAATAATGAGCC) (SEQ ID NO: 4).

30 The 1 kb PCR product was cloned into pGEM-T-Easy, resulting in the plasmid pGEM-Dmhsp70. The GUS gene was excised from the plasmid pGEM3Z-GUS[s] using the restriction endonuclease EcoRI, and ligated into the pGEM-Dmhsp70 plasmid, previously linearized with EcoRI. This ligation resulted in two plasmids, phspGUS[s], with the GUS gene in the sense 35 orientation with respect to the promoter, and phspGUS[a/s], with the GUS gene in the antisense orientation. A third plasmid, pHSP70GUS[i/r], was prepared

- that expressed an inverted repeat dsRNA specific to the GUS open reading frame (ORF), by ligating a 558 bp DNA fragment, representing the 5' end of the GUS gene, to the 3' end of the GUS ORF. The resulting coding sequence, when transcribed, could produce a transcript with complementary sequences at
- 5 the 5' and 3' ends, which could fold back upon itself to form a hairpin dsRNA, with double-stranded sequence for 558 bases.

#### *H. armigera vATPase in vitro transcription plasmids*

- A 386 bp segment of a putative vATPase gene was amplified from *H. armigera* genomic DNA using the two primers HaATP1f (CCGAAAATCCAATCTACGGACCC) (SEQ ID NO: 5) and HaATP1r (CGACGAATAACCTGGGCTGTTGC) (SEQ ID NO: 6). The primers were based on DNA sequence of a putative vATPase gene identified from a *H. armigera* EST clone that showed 97% sequence identity to the vATPase gene of *Heliothis virescens* (GenBank accession # L16884). The 386 bp product was amplified using a Perkin Elmer 2400 Thermocycler using the following PCR conditions: 1 cycle of 95°C for 5 min, 25 cycles of 95°C x 30 sec, 55°C x 30 sec, 72°C x 30 sec, and 1 cycle of 72°C x 10min, 25°C x 5min. The PCR product was ligated into the pGem-T-Easy cloning vector (Promega) in both orientations with respect to the T7 promoter, producing the plasmids pGEMHaATPase1[s] and pGEMHaATPase1[a/s], which were used to produce in vitro transcribed sense and antisense vATPase RNAs.

#### *Drosophila transformation*

- 25 The GUS gene encoding the bacterial enzyme  $\beta$ -glucuronidase, was inserted into the *P*-element transformation vector pCaSpeR-act, which placed the GUS gene under the control of the act5c promoter. The GUS gene was then introduced into the *Drosophila* germline by *P*-element transformation (Spradling and Rubin, 1982). Transformants were backcrossed to 30 chromosomal balancer strains to identify into which chromosome the transgene had inserted. Southern analyses of DNA from G2 flies were preformed to determine the copy number of the transgene in the GUS transgenic stock.

#### *Preparation of double-stranded RNA by in vitro transcription*

- 35 The plasmids pGEM3Z- $\Delta$ GUS[s] and - $\Delta$ GUS [a/s] were linearized using *Bam* HI. Sense and antisense RNA was prepared using T7 RNA polymerase

using Promega's RiboMAX Large Scale RNA Production System, according to the manufacturer's instructions. To produce dsRNA, sense and antisense RNAs were mixed in equimolar quantities and annealed for 10 minutes at 37°C. The RNA was extracted with phenol/chloroform and then chloroform,  
5 precipitated with ethanol, and resuspended in 10 mM Tris-HCl, pH 9. Formation of dsRNA was confirmed by resolving the annealed and non-annealed RNAs on a 1.0% agarose gel in TBE (90 mM Tris-borate, 2 mM EDTA, pH 8.0).

To produce vATPase dsRNA, the plasmids pGEMHaATPase1[s] and pGEMHaATPase1[a/s] were linearized with *Bam* HI and sense, antisense, and  
10 double-stranded RNAs were produced as described above.

#### *Embryo injections*

Preblastoderm *D. melanogaster* embryos were microinjected with DNA or RNA according to the method of Spradling and Rubin (1982) and *H. armigera* embryos were microinjected as previously described (Pinkerton et al.,  
15 1996). The embryos were injected with sense, antisense, and dsRNAs dissolved in injection buffer (5 mM KCl, 0.1 mM PO<sub>4</sub>, pH 6.8) at a concentration of 100 ng/ul. Approximately 50 pg of RNA were injected in each embryo. Negative control embryos were mock-injected with injection buffer alone.  
20 Embryos injected with DNA were injected with approximately 250 pg of plasmid DNA. The embryos were permitted to fully develop for 16 h, and were either snap frozen for use in subsequent GUS assays or were permitted to hatch and surviving larvae were transferred to vials containing culture medium. Individual larvae and adult insects were collected and snap frozen at -80°C.

25

#### *Oral dsRNA delivery*

Newly hatched 1<sup>st</sup> instar larvae (*Drosophila melanogaster* or *Helicoverpa armigera*) were transferred to 96-well plates in groups of 10 – 25, and washed in phosphate buffered saline (PBS). Sense, antisense, and annealed dsRNAs  
30 (0.05 – 2 ug) were mixed with 1 ul of transfection promoting agent, 0.5 mM spermidine or protamine sulphate (0.5 mg/mg DNA), in a volume of 20 ul of PBS or buffered sucrose (20% sucrose, 10 mM Tris, pH 7.5). After 30 min, red food dye was added to the transfection promoting agent-RNA mixture and the mixture was added to the neonate larvae. The larvae remained immersed in the  
35 mixtures for 1 h, and larvae were then transferred to rearing medium.

Approximately 90% of individuals treated in this manner contained red food dye in their guts, indicating that most had ingested the mixture.

#### *Rearing conditions*

- 5 *D. melanogaster* were raised at 25°C on standard yeast-agar *Drosophila* culture media (Roberts and Standen, 1998). *H. armigera* were raised as previously described (Duve et al., 1997).

#### *GUS assays*

- 10 Insects were homogenised in homogenisation buffer (50 mM NaHPO<sub>4</sub>, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100), and GUS enzyme activity was measured using 4-methylumbelliferyl β-D-glucuronic acid as a substrate in fluorometric assays as described (Gallagher, 1992). Protein assays were performed using the 15 Bradford assay (Bradford, 1976). Dissected insects were stained for GUS activity using 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-GlcU) as described (Naleway, 1992).

#### Results

- 20 *Characterisation of the GUS transgenic strain*

Standard genetic and Southern analyses confirmed that the GUS transgenic stock of *D. melanogaster* contained a single insertion of the act5c-GUS construct, located on chromosome III (results not shown). The GUS gene was constitutively expressed throughout the body, with extensive GUS activity observed in the fat body and gonads of both males and females (data not shown). Fluorometric GUS enzyme assays confirmed that all developmental stages of the GUS transgenics had at least 18 times more GUS activity than their non-transformed counterparts (Table 1).

- 30 *Silencing of the GUS gene in Drosophila embryos using in vitro transcribed and annealed double stranded RNA*

Following injection of RNA into preblastoderm embryos, embryos were permitted to develop for 16 h, just prior to hatching, before they were assayed for GUS activity. Embryos were pooled in groups of 25, whereas larvae and 35 adults were assayed individually for GUS activity. While it was not possible to determine which individual embryos were most affected by the RNA injections,

it was clear that both sense and antisense had no or little affect on GUS activity, whereas those embryos injected with dsRNA showed significant reductions in GUS activity (Table 2). Northern analyses of RNA from mock-injected and dsRNA-injected embryos confirmed that the reduction of GUS activity correlated with reduction of GUS transcripts in the dsRNA-injected embryos (results not shown). Interestingly, the silencing of the GUS gene expression persisted throughout development, as both larvae and adults that had been treated with dsRNA as embryos still showed substantial reductions in GUS activity. These results confirmed that GUS gene expression could be effectively reduced by direct delivery of *in vitro*-prepared dsRNA into the embryos.

**Table 1. GUS activity in non-transgenic and transgenic *D. melanogaster***

| Developmental stage          | GUS activity (pmol MU/min/individual) | Fold increase in GUS activity in transgenics |
|------------------------------|---------------------------------------|--|
|                              | Non-transgenic                        | Transgenic                                   |
| embryo                       | 74 ± 20                               | 1980 ± 152                                   |
| 3 <sup>rd</sup> instar larva | 417 ± 34                              | 7390 ± 780                                   |
| adult                        | 574 ± 55                              | 12620 ± 827                                  |

15

**Table 2. Reduction of GUS activity following embryonic injection of RNA to *D. melanogaster* GUS strain embryos. Values represent the percentage decrease (± standard error) of GUS activity relative to mock-injected embryos.**

|               | Reduction in GUS activity following delivery of RNA (%) |                     |                     |
|---------------|---|---------------------|---------------------|
|               | Embryos <sup>1</sup>                                    | Larvae <sup>2</sup> | Adults <sup>2</sup> |
| Sense RNA     | 2 ± 1   | 3 ± 2               | 5 ± 3               |
| Antisense RNA | 9 ± 4   | 7 ± 5               | 15 ± 8              |
| ds RNA        | 65 ± 14   | 41 ± 7              | 32 ± 5              |

<sup>1</sup> Values represent results from 3 separate replicates of 25 embryos each.

20 <sup>2</sup> Values represent results from 3 separate replicates of 10 individuals each.

*Silencing of the GUS gene in Drosophila embryos using in vivo-produced dsRNA*

GUS strain embryos were injected with the plasmids phspGUS[s], phspGUS[a/s], and phspGUS[i/r], and then heat shocked 6 h post injection. The 5 embryos were collected just prior to hatching (16 h development), and were assayed for GUS activity. The embryos injected with phspGUS[s] showed no difference in GUS activity, whereas embryos injected with phspGUS[a/s] showed a 12% decrease in GUS activity relative to mock-injected controls (Table 3). Embryos injected with the inverted repeat RNA expression construct, 10 phspGUS[i/r], showed substantial (90%) reduction of GUS activity. Adults that developed from embryos injected with the phspGUS[i/r] plasmid showed persistence of the gene silencing phenotype, having a 55% reduction in GUS activity relative to mock-injected controls. Adults derived from injections of plasmids that expressed sense or antisense RNA showed no persistence of the 15 gene silencing.

Table 3. Reduction of GUS activity following embryonic injection of RNA-expression plasmids to *D. melanogaster* GUS strain embryos. Values represent the percentage decrease ( $\pm$  standard error) of GUS activity relative to mock-injected embryos.

|              | Reduction in GUS activity (%) |            |
|--------------|-------------------------------|------------|
|              | Embryos                       | Adults     |
| phspGUS[s]   | 1 $\pm$ 1                     | 1 $\pm$ 2  |
| phspGUS[a/s] | 12 $\pm$ 2                    | 2 $\pm$ 1  |
| hspGUS[i/r]  | 90 $\pm$ 8                    | 55 $\pm$ 6 |

PCR analysis of different developmental stages showed that the injected plasmid could not be detected beyond first instar larvae (Figure 1), suggesting that the injected DNA was quickly degraded once the insects moulted into 2<sup>nd</sup> 25 instar larvae. The persistence of the gene silencing throughout development was therefore most likely due to the persistence of the dsRNA, and not due to sustained expression of dsRNA from the injected plasmid.

*Silencing of the GUS gene in Drosophila following soaking of larvae in dsRNA*  
30 Drosophila larvae fed naked GUS dsRNA showed no changes in GUS gene expression (results not shown). Similarly, no change in GUS activity in 2<sup>nd</sup>

instar larvae or adults was observed when neonate larvae were immersed in a DMRIE-C mixture containing GUS sense or antisense RNA (results not shown). In contrast, 15% of neonates soaked in transfection promoting agent containing GUS dsRNA developed into adult flies that showed >90% reduction of GUS 5 activity (Figure 2). Another 35% of the surviving flies showed an intermediate (20-80%) reduction of GUS expression. Similarly, 2<sup>nd</sup> instar larvae derived from neonates soaked in dsRNA showed a similar result, with 20% of larvae having >90% reduction of GUS activity, and another 40% of the larvae showing a reduction of GUS activity between 20% and 80% of normal GUS activity levels. 10 These results indicate that *in vitro* transcribed and annealed dsRNA can be fed to neonates and cause extensive, body-wide gene silencing of the target gene. This method of dsRNA delivery seems relatively benign, as no larvae were observed to die or suffer from the transfection promoting agent treatment. Gene silencing appears to be gene specific, as the insects showing reduced GUS 15 activity appeared healthy and showed no other observable phenotype.

As the larvae were soaking in the mixture, it is possible that entry of dsRNA may have occurred either by ingestion, perfusion into the trachea, or by absorption through the cuticle. However, a small percentage (10%) of surviving larvae were observed not to have any food colouring in their guts. These 20 individuals showed no reduction of GUS activity, which suggested that the primary route of entry for the dsRNA is via the alimentary canal (results not shown).

The concentration of dsRNA fed to the larvae correlated directly with the number of individuals that exhibited strong suppression of GUS activity. The 25 lowest concentration (0.25 ug/ml) of dsRNA tested, using DMRIE-C, produced 4/20 flies that displayed a reduction of GUS activity greater than 25% (Figure 3). In contrast, the highest concentration of dsRNA tested (1.0 ug/ml) produced 12/20 flies with a reduction of GUS activity greater than 25%. At this highest dose, the greatest number of flies (5/20) showed a reduction of GUS activity of 30 greater than 80%. While these sample sizes are small (20 individuals/treatment), they indicate that the extent of gene silencing may be dsRNA dose-dependent.

Lipofectamine, Cellfectin, and DMRIE-C (Life Technologies), each produced individuals with a measurable level of reduced GUS activity (Figure 35 4). DMRIE-C provided the greatest number of individuals with extensive gene silencing, with 25% of the larvae having greater than 75% of the GUS activity

eliminated. Two individuals out of 20 showed 100% gene silencing using this transfection promoting agent. Transfections with Lipofectamine and Cellfectin resulted in 26-50% silencing of the GUS gene in 35% of the larvae tested, which indicates that these transfection promoting agents could also serve to 5 deliver dsRNA to *Drosophila* via ingestion.

Given that most (approximately 70%, results not shown) of the GUS gene expression is found in the fat body and gonads, the silencing signal had obviously passed beyond the gut tissues and spread throughout the body. This gene silencing spreading phenomenon is not unlike that seen in *C. elegans* 10 nematodes fed dsRNA. However, it is surprising to observe gene silencing in the insect following this mode of delivery of dsRNA, as the gut of *Drosophila* is physically and physiologically more complex than that of *C. elegans*. Most notably, *Drosophila* produces a peritrophic membrane throughout the length of 15 the midgut, which theoretically could potentially reduce or prevent transmission of dsRNA to the midgut cells.

Addition of a nucleic acid condensing agent (spermidine or protamine sulfate) to the RNA mixture was found to enhance the efficiency of RNAi in *Drosophila*. Without adding spermidine, only 20% of treated larvae displayed a reduction of GUS activity greater than 20%, and only a maximum of 32% GUS 20 silencing was observed (Figure 5). Not only did the percentage of individuals with significant levels of GUS gene silencing increase by using spermidine, but the maximum level of GUS gene silencing increased to 100% in some individuals (see Figure 2). A similar enhancement of RNAi was observed if protamine sulphate was used instead of spermidine (results not shown).

25 The efficiency of RNAi in *Drosophila* was found to improve slightly when PBS was replaced with buffered sucrose during the mixing of the RNA with the transfection promoting agents (Table 4). Although it has not been examined further, it is anticipated that replacement of PBS with sucrose will improve efficiency of the packaging of the RNA in many of the transfection promoting 30 agents under consideration.

A selection of transfection promoting agents was kindly provided by Trevor Lockett and colleagues (CSIRO Molecular Science). These transfection promoting agents are thoroughly described in the patent "Delivery of Nucleic Acids" (PCT/AU95/00505, US 5,906,922). A comparison of 11 of these CSIRO 35 reagents with the 5 commercially available reagents was conducted, and many of the CSIRO liposomes were more effective at producing an RNAi effect in

- Drosophila* (Table 5). In particular, liposomes CS096, CS102, and CS129 performed better than the best-performing commercially available liposome, DMRIE-C. All of the CSIRO liposomes tested produced a greater number of individuals affected by RNAi than the poorest commercially available liposome, 5 DOTAP. These results confirm that optimised delivery of dsRNA to insects can be achieved by selecting appropriate transfection promoting agents.

Table 4. Percentage of 2<sup>nd</sup> instar larvae showing greater than a 25% reduction of GUS gene activity following soaking in transfection promoting agents that 10 were mixed with RNA in either PBS or buffered sucrose solutions. Values represent the mean and standard deviation from two replicates of 25 insects.

| Transfection Promoting Agent | PBS    | Buffered Sucrose |
|------------------------------|--------|------------------|
| DMRIE-C                      | 60 ± 7 | 72 ± 10          |
| Lipofectamine                | 35 ± 6 | 49 ± 8           |
| DOTAP                        | 0 ± 0  | 5 ± 3            |

*Silencing of an endogenous gene in H. armigera*

Neonate *H. armigera* were soaked in a composition containing 15 transfection promoting agent and dsRNA specific to a putative vacuolar ATPase gene. Several vATPase genes are present in Lepidoptera, some of which are known to encode subunits of proton pumps in the midgut cells. These proton pumps are responsible for establishing and maintaining the high pH (approximately pH 10) environment of the lepidopteran midgut.

20 While all *Drosophila* larvae survived the soaking treatment, only 64 % of *H. armigera* larvae were alive 24h after exposure to transfection promoting agent containing no RNA (Table 6). A similar percentage of caterpillars (62 %) survived a treatment containing transfection promoting agent mixed with GUS dsRNA. Only 40 % of larvae soaked in transfection promoting agent mixed with 25 vATPase dsRNA survived the first 24 h. In addition to a slightly reduced survival after the first 24 hours, delayed development was also observed for larvae exposed to vATPase dsRNA.

Of those larvae surviving beyond 24 h, 85 % of the control larvae reached pupation by day 10. In contrast, only 40 % of surviving larvae treated 30 with vATPase dsRNA pupated by day 10. The overall mortality for larvae treated with vATPase dsRNA, relative to those treated with transfection

promoting agent alone was 52%. Larvae treated with GUS dsRNA were not significantly affected, as 82 % had pupated by day 10. Oral delivery of vATPase dsRNA therefore resulted in both reduced survival and delayed development in *H. armigera* larvae.

5

Table 5. Ordered ranking of CSIRO transfection promoting agents and commercially available transfection promoting agents in their ability to induce RNAi of the GUS transgene in *Drosophila*. The percentage of 2<sup>nd</sup> instar larvae having greater than a 25% reduction in GUS activity was determined after 10 neonate larvae were soaked in the transfection promoting agent containing GUS dsRNA in buffered sucrose. Values represent the mean and standard deviation from two experiments with 15 insects each.

| Ranking | Transfection Promoting Agent | % 2 <sup>nd</sup> instar larvae with >25% GUS RNAi |
|---------|------------------------------|--|
| 1       | CS096                        | 70 ± 5   |
| 2       | CS102                        | 63 ± 14  |
| 2       | CS129                        | 63 ± 5   |
| 3       | DMRIE-C                      | 56 ± 14  |
| 4       | CS078                        | 46 ± 9   |
| 5       | CS051                        | 43 ± 14  |
| 5       | CS027                        | 43 ± 5   |
| 6       | CS041                        | 40 ± 9   |
| 7       | Lipofectamine                | 36 ± 5   |
| 8       | CS042                        | 23 ± 5   |
| 9       | Cellfectin                   | 20 ± 9   |
| 9       | CS060                        | 20 ± 9   |
| 10      | Lipofectin                   | 16 ± 5   |
| 11      | CS039                        | 10 ± 5   |
| 11      | CS015                        | 10 ± 5   |
| 12      | DOTAP                        | 3 ± 5  |

\* Complete names of the transfection promoting agents are provided in the "Transfection Promoting Agent" section of the Detailed Description. Lipofectin, 15 Lipofectamine, Cellfectin, and DMRIE-C were obtained from Life Technologies, whereas DOTAP was obtained from Boehringer Mannheim.

- Little is known about the expression of the particular vATPase gene that was targeted, other than that it is expressed in gut tissues (as it was isolated from a gut-specific EST library). It is not presently known if the targeted
- 5 vATPase gene is also expressed elsewhere in the body, nor if the extent of gene silencing was sufficient to reduce the majority of vATPase activity. Nevertheless, the GUS dsRNA produced no deleterious effect on the caterpillars, which indicates that the vATPase dsRNA-mediated gene silencing was sufficiently effective to cause a significant level of mortality and morbidity.
- 10 Unlike *D. melanogaster*, the use of Lipofectamine provided the best RNAi (Table 7). As with *Drosophila*, treatments of RNA alone or RNA with spermidine failed to result in observable RNAi.

15 Table 6. Effects of soaking *H. armigera* larvae in transfection promoting agent containing dsRNA. The results represent the mean and standard errors for three separate experiments using 20 larvae for each treatment.

|                                    |           | % surviving larvae at 24 h <sup>a</sup> | % pupated by day 10 <sup>b</sup> | % survival to adulthood <sup>c</sup> |
|------------------------------------|-----------|---|----------------------------------|--------------------------------------|
| Transfection Agent alone           | Promoting | 64 ± 5                                  | 85 ± 5                           | 100                                  |
| Transfection Agent + GUS dsRNA     | Promoting | 60 ± 6                                  | 82 ± 6                           | 91                                   |
| Transfection Agent + vATPase dsRNA | Promoting | 40 ± 12                                 | 40 ± 5                           | 52                                   |

a – based on three experiments using 20 insects each

b – percentage based on those insects surviving past 24 h post treatment

c- percentage survival relative to the transfection promoting agent treated controls.

#### Feeding RNA extracts from insects that produce dsRNA

RNA was extracted from a group of 100 flies that had been injected as embryos with the phspGUS[i/r] plasmid. The injected embryos had been subjected to a single heat shock to produce GUS dsRNA during mid embryogenesis. As no plasmid DNA could be detected in developmental stages beyond 1<sup>st</sup> instars, it is not expected that further RNA would be transcribed from this template DNA. The extracted RNA was injected into

embryos at a concentration of 1 ug/ul and the embryos were later assayed for GUS activity. GUS activity was reduced by 40% in these embryos, which indicates that the dsRNA is both extractable and still capable of promoting gene silencing when transferred back into naïve insects. RNAs obtained from flies 5 previously injected with either the phspGUS[s] plasmid (sense RNA) or phspGUS[a/s] plasmid (antisense RNA) were also injected into embryos, and these embryos showed no change in GUS activity (results not shown).

Table 7. Comparison of transfection promoting agent efficiencies at producing 10 RNAi-induced delayed development in *H. armigera*. Values represent the percentage of larvae that survived the first 12 h post-treatment that reached pupation by day 10. Six replicates of 10 larvae were tested for each of the conditions.

| RNA mixture                                  | % pupation by day 10 |
|--|----------------------|
| Buffer only                                  | 87 ± 5               |
| RNA + buffer                                 | 84 ± 4               |
| RNA + buffer + spermidine                    | 82 ± 6               |
| RNA + buffer + spermidine + DOTAP            | 73 ± 7               |
| RNA + buffer + spermidine + Lipofectin       | 69 ± 10              |
| RNA + buffer + spermidine + Lipofectamine    | 48 ± 16              |
| RNA + buffer + spermidine + Cellfectin       | 54 ± 9               |
| RNA + buffer + spermidine + DMRIE-C          | 53 ± 14              |
| RNA + buffer (no spermidine) + Lipofectamine | 58 ± 8               |

15 The RNA extracted from flies previously injected with the phspGUS[i/r] plasmid was then mixed with DMRIE-C and fed to neonate larvae. Developed larvae and adults were assayed for GUS activity, and 30% of the 3<sup>rd</sup> instar larvae and 20% of the adults showed between 25 and 50% reduction in GUS activity (Figure 6). These results indicate that dsRNA can be fed to neonates 20 not only as *in vitro* transcribed and annealed full length inverted repeat dsRNA, but also as dsRNA that has been processed within the insect. Although the proportion of dsRNA relative to the total RNA extracted was not determined, the quantity of dsRNA extracted from the insects was obviously sufficient to promote gene silencing in the fed neonates.

Discussion

The present inventors have demonstrated that dsRNA can be delivered to arthropods. Direct feeding of naked, unpackaged, dsRNA failed to produce an RNAi phenotype in *D. melanogaster* or *H. armigera*, indicating that the 5 transfection promoting agents were necessary for effective transfection in these species. However, it is envisaged that in arthropods with a simple digestive system naked dsRNA may be effective in obtaining gene silencing.

Notably, the same transfection promoting agents were effective at delivering dsRNA in *D. melanogaster* and *H. armigera*, despite the pH 10 differences in the guts of these two species.

A significant finding was that dsRNA that had been previously processed within one arthropod could still facilitate RNAi in another arthropod, even when the RNA was purified from its associated proteins. It is anticipated that the purification process would remove all dsRNA-associated proteins, such as the 15 so-called dicer proteins, which are believed to mediate target RNA degradation. Assuming that the majority of the dsRNA purified from the arthropods, and subsequently ingested by the neonates was the processed 21- and 22-mer oligonucleotides, it appears that the effective functional unit in the latter experiment is the short oligonucleotides. However, longer lengths of dsRNA are 20 clearly effective once ingested, as evidenced by the ingestion of in vitro transcribed GUS and vATPase dsRNAs.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the 25 specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

All publications discussed above are incorporated herein in their entirety.

Any discussion of documents, acts, materials, devices, articles or the like 30 which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed, particularly in Australia, before the priority date of each claim of this 35 application.

References

- Allen, T.M. and Chonn, A. (1987) FEBS Lett. 223:42-46.
- 5 Bosher, J.M., and Labouesse, M. (2000) Nature Cell Biol 2: E31-E36.
- Bradford, M.M. (1976) Anal. Biochem. 72: 248-54.
- Cameron, F.H., Moghaddam, M.J., Bender, V.J., Whittaker, R.G., Mott, M.,  
10 Lockett, T.J. (1999) Biochim. Biophys. Acta /Biomembranes. 1417: 37-50.
- Dougherty, W.G. and Parks, T.D. (1995) Curr. Opin. Cell Biol. 7:399-405.
- Duve, H., Johnsen, A.H., Maestro, J.L., Scott, A.G., Winstanley, D., Davey, M.,  
15 East, P.D, Thorpe, A. (1997) Peptides 18: 1301-1309.
- Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Nature 411: 494-498.
- 20 Gallagher, S. 1992 Quantitation of GUS activity by fluorometry. In: GUS Protocols (ed S. Gallagher). Academic Press Inc. San Diego.
- Gawron-Burke, C. and Baum, J.A. 1991. Genetic manipulation of *Bacillus thuringiensis* insecticidal crystal protein genes in bacteria p. 237-263 In J.K.  
25 Setlow (ed.) Genetic engineering: principles and methods, vol 13 Plenum Press, New York.).
- Maeda I., Kohara, Y., Yamamoto, M., and Sugimoto, A. (2001) Current Biology 11: 171-176.
- 30 Naleway, J. 1992 Histochemical, spectrophotometric, and fluorometric GUS substrates. In: GUS Protocols (ed S. Gallagher). Academic Press Inc. San Diego.
- 35 Needleman, S.B. and Wunsch, C.D. (1970) J. Mol. Biol. 48:443-453.

- Pinkerton, A.C., O'Brochta, D., and Atkinson, PW. (1996) Insect Molec. Biol. 5: 223-227.
- Roberts, D.B. and Standen, G.N. (1998) The elements of *Drosophila* biology and genetics. In: *Drosophila: A Practical Approach* (ed. D.B. Roberts) Oxford University Press, Oxford.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual. Cold Springs Harbor Laboratory, New York.
- 10 Spradling, A.C., and Rubin, G.M. (1982) Science 218: 341-347.
- Tabara, H., Grishok, A., and Mello, C.C. (1998) Science 282: 430-431.
- 15 Thummel, C.S., Boulet, A.M., Lipshitz, H.D. 1988. Gene 74: 445-456
- Timmons, L. and Fire, A. (1998) Nature 395: 854.
- Waterhouse, P.M., Graham, M.W., and Wang, M.-B. (1998) Proc. Natl. Acad. Sci. 95: 13959-13964.
- 20 25 Waterhouse, P.M., Wang, M.-B., Lough, T. (2001) Nature 411: 834-842.
- White, 1988. The anatomy. In: The nematode *Caenorhabditis* (W.B. Wood ed.) Cold Spring Harbor Press, NY
- Wu, N.Z., Da, D., Rudoll, T.L., Needham, D., Whorton, A.R. and Dewhirst, M.W. (1993) Cancer Res. 53:3765-3770.

## CLAIMS:

1. A method of determining the biological function of a target RNA in an arthropod comprising delivering to the arthropod a dsRNA molecule which specifically reduces the level of the target RNA and/or the production of a protein encoded by the target RNA in a cell of the arthropod, and assessing the effect of the dsRNA on at least one biological function of the arthropod.
- 10 2. The method of claim 1, wherein the dsRNA is delivered by a process comprising contacting the arthropod with the dsRNA.
- 15 3. The method of claim 2, wherein said contacting comprises wholly or partially soaking the arthropod in a composition comprising the dsRNA.
4. The method of claim 1, wherein the dsRNA is delivered by a process comprising feeding the dsRNA to the arthropod.
- 20 5. The method according to any one of claims 1 to 4, wherein the dsRNA is delivered in a composition comprising a transfection promoting agent.
6. The method of claim 5, wherein the transfection promoting agent is a lipid-containing compound.
- 25 7. The method of claim 6, wherein the lipid-containing compound is selected from the group consisting of; Lipofectamine, Cellfectin, DMRIE-C, DOTAP and Lipofectin.
8. The method of claim 6, wherein the lipid-containing compound is a Tris cationic lipid.
- 30 9. The method of claim 8, wherein the Tris cationic lipid is selected from the group consisting of: CS096, CS102, CS129, CS078, CS051, CS027, CS041, CS042, CS060, CS039, and CS015.

10. The method according to any one of claims 5 to 9, wherein the composition further comprises a nucleic acid condensing agent.
- 5 11. The method of claim 10, wherein the nucleic acid condensing agent is selected from the group consisting of: spermidine and protamine sulfate.
12. The method of claim 1, wherein the dsRNA is delivered by a process comprising feeding a transgenic organism expressing the dsRNA to the arthropod.
- 10 13. The method of claim 12, wherein the transgenic organism is a transgenic plant.
14. The method of claim 1, wherein the dsRNA is delivered by a process comprising contacting the arthropod with a virus expressing the dsRNA.
- 15 15. The method according to any one of claims 1 to 14, wherein the dsRNA comprises a nucleotide sequence having at least 90% identity to at least a portion of the sequence of the target RNA.
- 20 16. The method according to any one of claims 1 to 14, wherein the dsRNA comprises a nucleotide sequence having at least 99% identity to at least a portion of the sequence of the target RNA.
- 25 17. The method according to any one of claims 1 to 16, wherein the arthropod is an insect.
18. A composition comprising dsRNA and a transfection promoting agent, wherein said dsRNA comprises a nucleotide sequence that is at least 90% identical to the sequence of a target RNA, wherein the target RNA is selected from the group consisting of: a naturally-occurring arthropod RNA, a naturally-occurring RNA of an organism that is a pathogen carried by an arthropod, a naturally-occurring RNA of a virus that infects an arthropod, an RNA copy of a naturally-occurring DNA virus that infects an arthropod, and a naturally-occurring RNA of a bacterium that infects an arthropod.
- 30
- 35

19. The composition of claim 18, wherein the transfection promoting agent is a lipid-containing compound.
- 5 20. The composition of claim 19, wherein the lipid-containing compound is selected from the group consisting of; Lipofectamine, Cellfectin, DMRIE-C, DOTAP and Lipofectin.
- 10 21. The composition of claim 19, wherein the lipid-containing compound is a Tris cationic lipid.
22. The composition of claim 21, wherein the Tris cationic lipid is selected from the group consisting of: CS096, CS102, CS129, CS078, CS051, CS027, CS041, CS042, CS060, CS039, and CS015.
- 15 23. The composition according to any one of claims 18 to 22, wherein the composition further comprises a nucleic acid condensing agent.
- 20 24. The composition of claim 23, wherein the nucleic acid condensing agent is selected from the group consisting of: spermidine and protamine sulfate.
- 25 25. The composition according to any one of claims 18 to 24, wherein the composition is formulated such that it can be applied to an area inhabited by a population of arthropods.
26. The composition according to any one of claims 18 to 25, wherein the composition further comprises an agriculturally acceptable carrier.
- 30 27. The composition according to any one of claims 18 to 26, wherein the composition is formulated as a bait.
28. A method of controlling an arthropod pest comprising delivering to the arthropod dsRNA by a process comprising contacting the arthropod with said dsRNA or feeding said dsRNA to the arthropod, for a time and under conditions sufficient for said dsRNA, or a degradation product

thereof, to specifically reduce the level of a target RNA and/or the production of a protein encoded by the target RNA in a cell of the arthropod, wherein the target RNA or the protein is important for arthropod survival, development and/or reproduction.

5

29. The method of claim 28, wherein the dsRNA is delivered in a composition according to any one of claims 18 to 27.
30. A method for controlling a pathogen transmitted by an arthropod, the method comprising delivering to the arthropod dsRNA by a process comprising contacting the arthropod with said dsRNA or feeding said dsRNA to said arthropod, for a time and under conditions sufficient for said dsRNA, or a degradation product thereof, to specifically reduce the level of a target RNA and/or the production of a protein encoded by the target RNA in a cell of the pathogen, wherein the target RNA or the protein is important for pathogen survival, development and/or reproduction.
31. The method of claim 30, wherein the dsRNA is delivered in a composition according to any one of claims 18 to 27.
32. The method of claim 30 or claim 31, wherein the pathogen is selected from the group consisting of: fungi, protozoans, bacteria and viruses.
33. A method of protecting an arthropod against a pathogen, parasite or predatory organism, the method comprising delivering to the arthropod dsRNA by a process comprising contacting the arthropod with said dsRNA or feeding said dsRNA to said arthropod, for a time and under conditions sufficient for said dsRNA, or a degradation product thereof, to specifically reduce the level of a target RNA and/or the production of a protein encoded by the target RNA in a cell of the pathogen, parasite or predatory organism, wherein the target RNA or the protein is important for the survival, development and/or reproduction of the pathogen, parasite or predatory organism.

35

34. The method of claim 33, wherein the dsRNA is delivered in a composition according to any one of claims 18 to 27.
35. A transgenic organism comprising a heterologous nucleic acid(s) which is transcribed to produce a dsRNA, wherein the portion of the dsRNA which is double stranded is about 21 to about 50 base pairs in length.
36. The transgenic organism of claim 35, wherein the dsRNA comprises a nucleotide sequence having at least 90% identity to at least a portion of the sequence of a target RNA selected from the group consisting of: a naturally-occurring arthropod RNA, a naturally-occurring RNA of an organism that is a pathogen carried by an arthropod, a naturally-occurring RNA of a virus that infects an arthropod, an RNA copy of a naturally-occurring DNA virus that infects an arthropod, and a naturally-occurring RNA of a bacterium that infects an arthropod.
37. The transgenic organism of claim 35 or claim 36, wherein the portion of the dsRNA which is double stranded is about 21 to about 23 base pairs in length.
38. The transgenic organism according to any one of claims 35 to 37, wherein the organism is selected from the group consisting of: plants and arthropods.

1/6

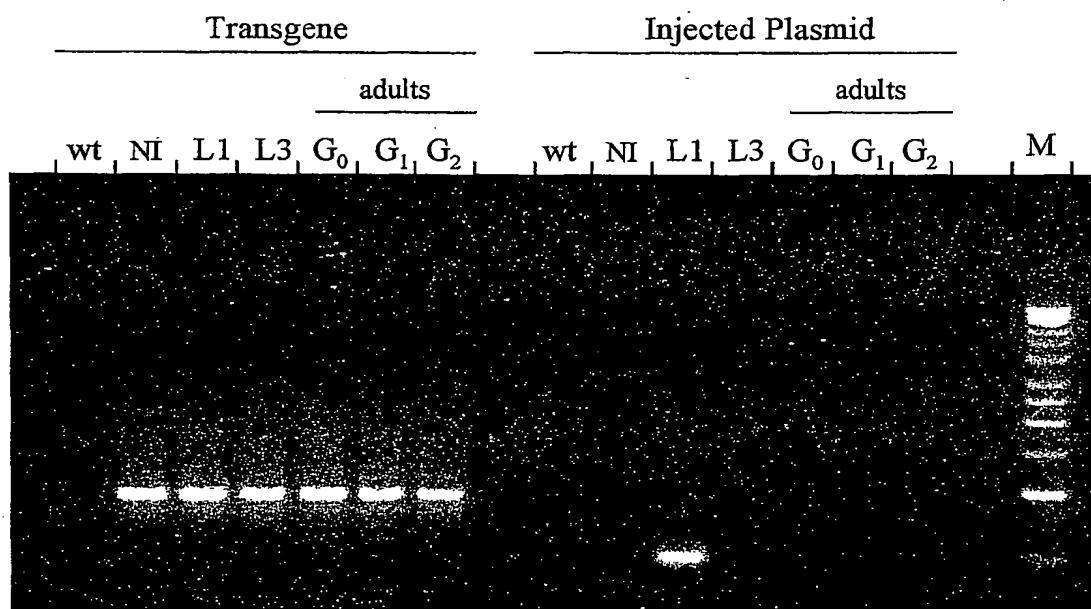


Figure 1

2/6

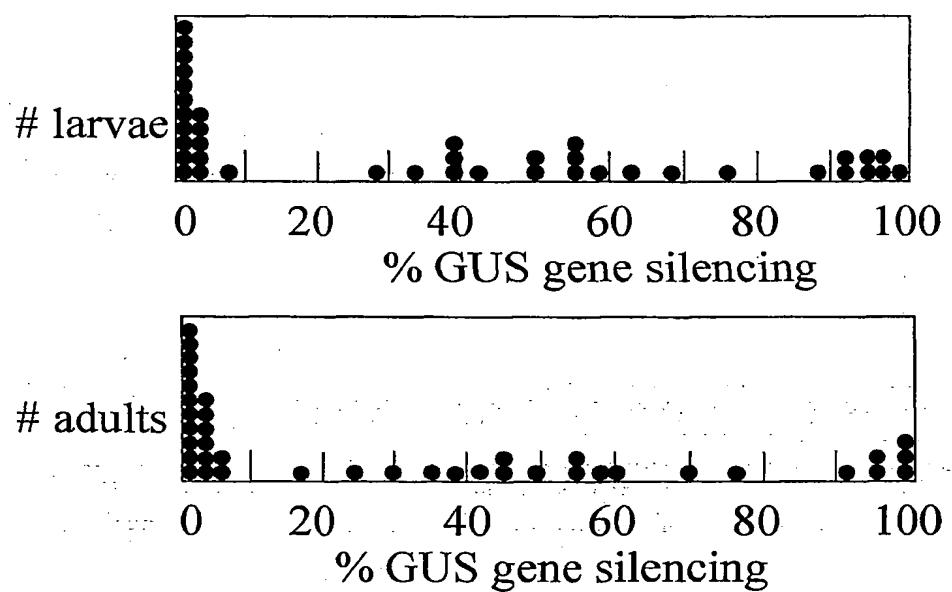


Figure 2

3/6

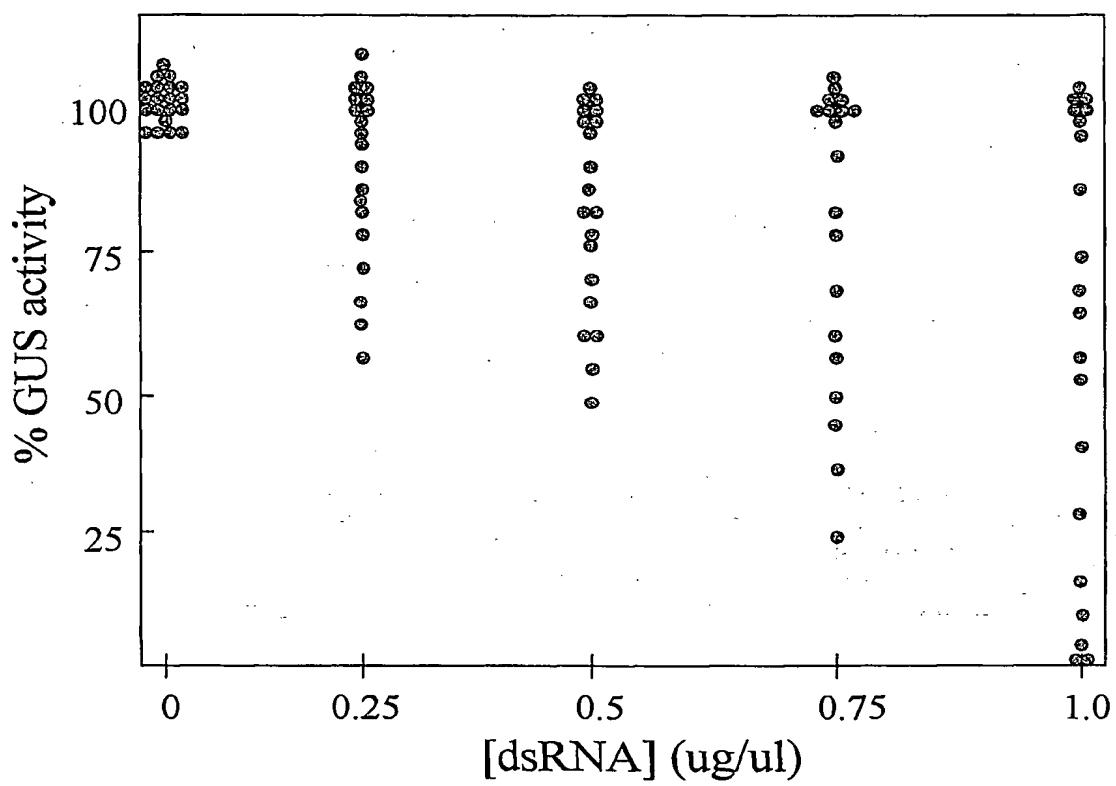


Figure 3

4/6

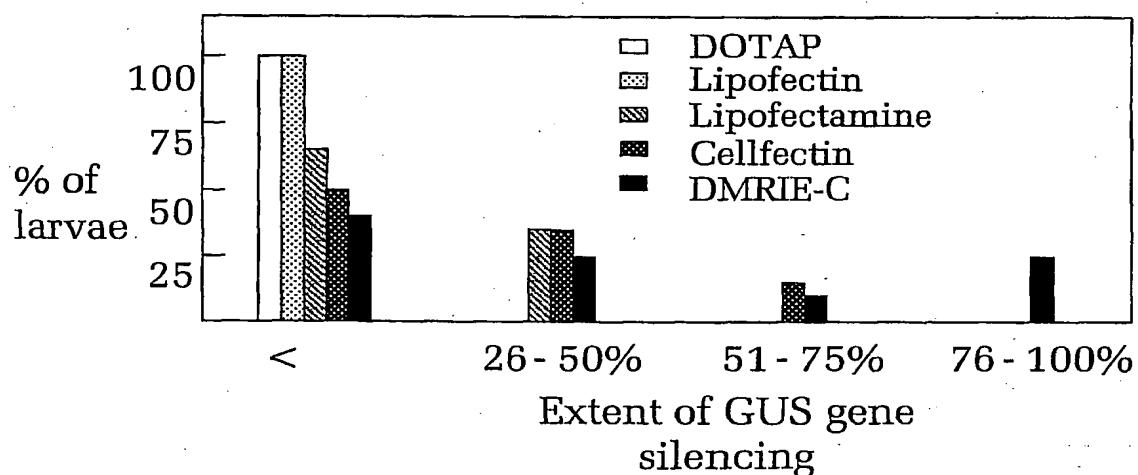


Figure 4

5/6

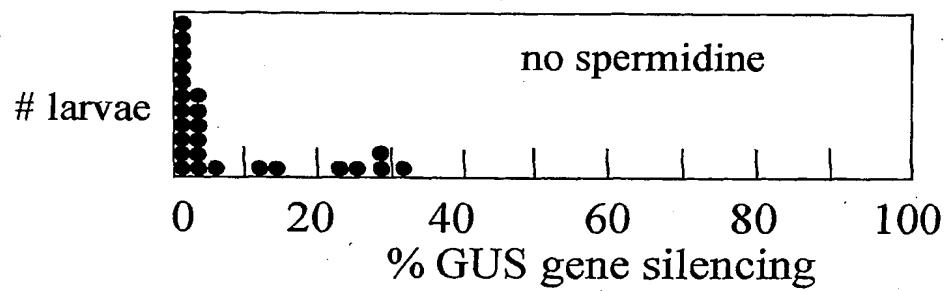


Figure 5

6/6

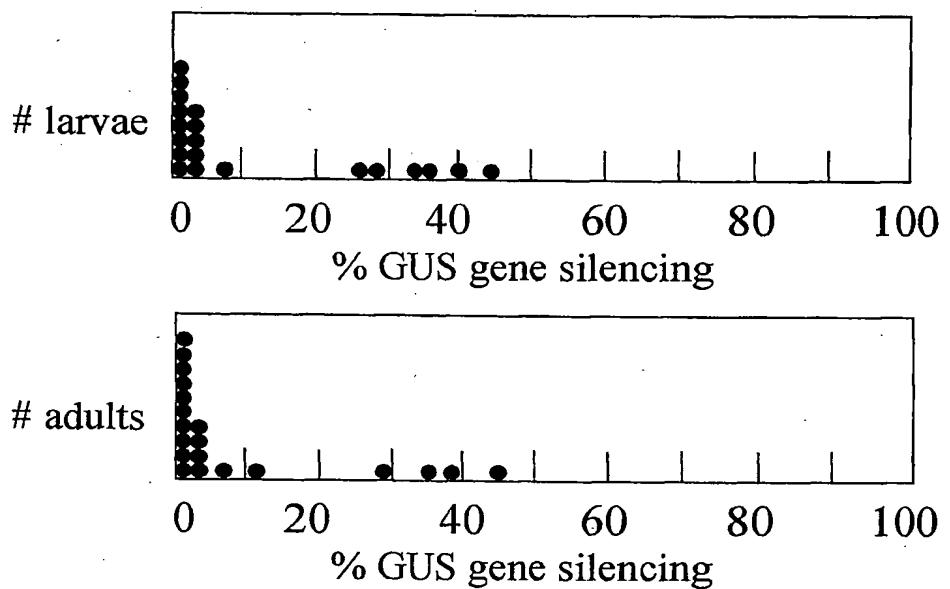


Figure 6

## SEQUENCE LISTING

<110> Commonwealth Scientific and Industrial Research Organisation

<120> Delivery of dsRNA

<130> 500689.

<150> AU PR 6215

<151> 2001-07-06

<160> 6

<170> PatentIn version 3.1

<210> 1

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 1

gaattcatgg tccgtcctgt agaaacc

27

<210> 2

<211> 24

<212> DNA

<213> Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR primer

&lt;400&gt; 2

gaattcccc accgaggctg tagc

24

&lt;210&gt; 3

&lt;211&gt; 27

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR primer

&lt;400&gt; 3

gaattctaga atcccaaaac aaactgg

27

&lt;210&gt; 4

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR primer

&lt;400&gt; 4

ggatcctgac cgtccatcg aataaaatga gcc

33

&lt;210&gt; 5

&lt;211&gt; 23

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR primer

<400> 5  
ccgaaaatcc aatctacgga ccc

23

<210> 6

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 6

cgacgaataa cctgggctgt tgc

23

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU02/00897

## A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. 7: C12N 15/11

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

## SEE ELECTRONIC DATABASES BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

## SEE ELECTRONIC DATABASES BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIDS CA MEDLINE BIOSIS AGRICOLA: double stranded RNA, dsRNA, RNAi, gene silencing, ptgs, arthropod, insect, drosophila, lipofection, cationic lipid.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No.       |
|-----------|--|-----------------------------|
| P, X      | WO 01/49844 A1 (RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY). OPI 12 July 2001. EPD 30 December 1999<br>Whole document particularly p11 and 31-32  | 18-24<br>35-38              |
| X         | WO 00/32779 A1 (LEPTIN, Martin). OPI 8 June 2000. EPD 30 November 1998.<br>Pp 26-28  | 1, 2, 16, 17,<br>35-38      |
| X         | Goto A, Matsuhashima Y, Kadokawa T, Kitagawa Y. <i>Drosophila</i> mitochondrial transcription factor A ( <i>d</i> -TFAM) is dispensable for the transcription of mitochondrial DNA in Kc167 cells. <i>Biochem J.</i> March 2001. 354: 243-248.<br>Whole document, particularly pp244 and 246 | 1-3, 5-11, 16-<br>24, 35-38 |

 Further documents are listed in the continuation of Box C See patent family annex

|       |   |     |  |
|-------|---|-----|--|
| * "A" | Special categories of cited documents:<br>document defining the general state of the art which is not considered to be of particular relevance                      | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  |
| "B"   | earlier application or patent but published on or after the international filing date   | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone   |
| "L"   | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "O"   | document referring to an oral disclosure, use, exhibition or other means  | "&" | document member of the same patent family  |
| "P"   | document published prior to the international filing date but later than the priority date claimed  |     |  |

Date of the actual completion of the international search  
18 September 2002

Date of mailing of the international search report

12 AUG 2002

Name and mailing address of the ISA/AU  
AUSTRALIAN PATENT OFFICE  
PO BOX 200, WODEN ACT 2606, AUSTRALIA  
E-mail address: pct@ipaaustralia.gov.au  
Facsimile No. (02) 6285 3929

Authorized officer

Gillian Allen  
Telephone No : (02) 6283 2266

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU02/00897

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |   |                         |
|---|---|-------------------------|
| Category*   | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No.   |
| X   | Misquitta, L.; Paterson, B.M. Targeted disruption of gene function in <i>Drosophila</i> by RNA interference (RNA-i): a role for nautilus in embryonic somatic muscle formation. <i>Proceedings of the National Academy of Sciences of the United States of America</i> , Feb 16, 1999. Vol. 96, No. 4. p. 1451-1456.<br>Whole document, particularly Abstract.  | 1, 2, 16, 17,<br>35-38. |
| X   | Piccin, Alberto; Salameh, Ahmad; Benna, Clara; Sandrelli, Federica; Mazzotta, Gabriella; Zordan, Mauro; Rosato, Ezio; Kyriacou, Charalambos P.; Costa, Rodolfo. Efficient and heritable functional knock-out of an adult phenotype in <i>Drosophila</i> using a GAL4-driven hairpin RNA incorporating a heterologous spacer. <i>Nucleic Acids Research</i> (2001), 29(12), e55/1-e55/5.<br>Whole document, particularly Abstract. | 1, 2, 16, 17,<br>35-38  |
| X   | Caplen N J; Fleenor J; Fire A; Morgan R A. dsRNA-mediated gene silencing in cultured <i>Drosophila</i> cells: a tissue culture model for the analysis of RNA interference. <i>GENE</i> , (2000 Jul 11) 252 (1-2) 95-105.<br>Whole document, particularly Abstract.  | 1, 2, 16, 17,<br>35-38  |
| X   | Kermerdell JR, Carthew RW. Heritable gene silencing in <i>Drosophila</i> using double stranded RNA. <i>Nature Biotechnology</i> . 2000. 18: 896-898<br>Whole document.  | 1, 2, 16, 17,<br>35-38  |
| X   | Carthew RW. Gene silencing by double-stranded RNA. <i>Curr Opin Cell Biol</i> . Apr 2001. 13(2): 244-8.<br>Particularly p 245   | 1, 2, 16, 17,<br>35-38  |
| A   | Zamore PD. RNA interference: listening to the sound of silence. <i>Nat Struct Biol</i> Sept 2001. 8(9): 746-50  |                         |
| A   | Vance V, Vaucheret H. RNA silencing in plants - defense and counterdefense. <i>Science</i> June 2001. 292(5525): 2277-80  |                         |

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/AU02/00897

**Box I Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos :  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos :Part 1, 18, 28 30, 33, 35-38  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
The search was primarily directed towards transformation of insects/arthropods by ds RNA by direct feeding of the RNA in combination with a transfection agent, as these are deemed to be the technical features that define the invention. The above independent claims are not limited to these technical features, and have not been fully searched..
  
3.  Claims Nos :  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

**Box II Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

**PCT/AU02/00897**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in<br>Search Report | Patent Family Member        |
|---|-----------------------------|
| WO 01/49844                               | AU 26255/01                 |
| WO 00/32779                               | AU 19688/00<br>US 6 135 942 |

END OF ANNEX

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
7 December 2006 (07.12.2006)

PCT

(10) International Publication Number  
WO 2006/129204 A3

(51) International Patent Classification:

C12N 15/11 (2006.01) A01P 7/04 (2006.01)  
A61K 31/713 (2006.01) C12N 1/21 (2006.01)  
A01N 63/00 (2006.01) C07K 14/435 (2006.01)

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(21) International Application Number:

PCT/TB2006/002360

(22) International Filing Date: 31 May 2006 (31.05.2006)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

05253339.5 31 May 2005 (31.05.2005) EP  
60/685,765 31 May 2005 (31.05.2005) US

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant (for all designated States except US): DE-  
VGEN NV [BE/BE]; Technologiepark 30, Zwijnaarde,  
B-9052 Gent (BE).

Published:

— with international search report

(72) Inventor; and

(75) Inventor/Applicant (for US only): KALETTA, Titus Jan  
[DE/BE]; Boomgaardstraat 1, B-9820 Merelbeke (BE).

(88) Date of publication of the international search report:  
21 June 2007

(74) Agents: BALDOCK, Sharon, Claire et al.; BOULT  
WADE TENNANT, Verulam Gardens, 70 Gray's Inn  
Road, London WC1X 8BT (GB).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2006/129204 A3

(54) Title: RNAI FOR CONTROL OF INSECTS AND ARACHNIDS

(57) Abstract: The present invention describes a new non-compound based approach for insect and/or arachnid control. The present inventors have identified for the first time novel targets for RNAi, which can effectively control insect and/or arachnid pest populations. Accordingly, the invention provides both nucleotide and amino acid sequences for the novel targets. Also provided are RNA constructs including double stranded RNA regions for mediating RNAi in insects, DNA constructs, expression vectors, host cells and compositions for controlling insects and/or arachnids using RNAi. Finally, the invention also provides for the use of the constructs, vectors, host cells and compositions in control of insects and/or arachnids populations and suitable kits for use in an RNAi based method of controlling insect and/or arachnid pests.

## INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2006/002360

A. CLASSIFICATION OF SUBJECT MATTER  
INV. C12N15/11 A61K31/713 A01N63/00 A01P7/04 C12N1/21  
C07K14/435

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N A61K A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, Sequence Search, WPI Data, PAJ, EMBASE, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|-----------|---|-----------------------|
| X         | <p>DATABASE EMBL [Online]<br/>26 February 2005 (2005-02-26),<br/>"USDA-FP_133348 5th Instar Glassy-winged<br/>Sharpshooter Homalodisca coagulata cDNA<br/>clone WHHC5-010_A12 5', mRNA sequence."<br/>XP002412194<br/>retrieved from EBI accession no.<br/>EM EST:DN196635<br/>Database accession no. DN196635<br/>the whole document</p> <p>-----</p> <p>-/-</p> | 1,7-10                |

 Further documents are listed in the continuation of Box C. See patent family annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority, claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

19 December 2006

20.03.2007

Name and mailing address of the ISA/  
European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Andres, Serge

## INTERNATIONAL SEARCH REPORT

 International application No  
 PCT/IB2006/002360

## C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|-----------|---|-----------------------|
| X         | DATABASE EMBL [Online]<br>11 September 2001 (2001-09-11), "SD19884.5prime SD Drosophila melanogaster Schneider L2 cell culture pOT2 Drosophila melanogaster cDNA clone SD19884 5 similar to RpS4: FBan0011276 GO:[protein biosynthesis (GO:0006412); cytosolic small ribosomal (40S)-subunit (GO:0005843); ribosomal protein (GO:0003735) ; ...."<br>XP002347921<br>retrieved from EBI accession no.<br>EM PRO:BI637800<br>Database accession no. BI637800<br>the whole document<br>----- | 1,7-10                |
| X         | DATABASE EPO Proteins [Online]<br>2 February 2004 (2004-02-02), "Sequence 23601 from Patent WO0171042."<br>XP002412195<br>retrieved from EBI accession no.<br>EPOP:CQ595843<br>Database accession no. CQ595843<br>the whole document<br>-& WO 01/71042 A2 (PE CORP NY [US])<br>27 September 2001 (2001-09-27)<br>-----  | 73,74                 |
| X         | DATABASE EMBL [Online]<br>6 April 2005 (2005-04-06), "Lysiphlebus testaceipes ribosomal protein S4 (RpS4) mRNA, complete cds."<br>XP002412196<br>retrieved from EBI accession no.<br>EM INV:AY961528<br>Database accession no. AY961528<br>the whole document<br>-----  | 1,7-10                |
| X         | DATABASE EMBL [Online]<br>14 October 1998 (1998-10-14), "EST284 Manduca sexta male antennae Uni-ZAP XR library Manduca sexta cDNA clone pMsmaD64 3' similar to 40S ribosomal protein S4, mRNA sequence."<br>XP002347922<br>retrieved from EBI accession no.<br>EM PRO:AI187495<br>Database accession no. AI187495<br>the whole document<br>-----  | 1,7-10                |
| A         | WO 2004/049807 A (EXOSECT LIMITED; HOWSE, PHILIP; UNDERWOOD, KAREN)<br>17 June 2004 (2004-06-17)<br>cited in the application<br>the whole document<br>-----   | 1-68,<br>70-76        |
|           |   | -/-                   |

## INTERNATIONAL SEARCH REPORT

|   |
|---|
| International application No<br>PCT/IB2006/002360 |
|---|

## C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|-----------|--|-----------------------|
| A         | WO 02/46432 A (AVENTIS CROPSCIENCE GMBH; GUNKEL, NIKOLAS) 13 June 2002 (2002-06-13)<br>the whole document<br>-----                                       | 1-68,<br>70-76        |
| A         | WO 2005/047300 A (UNIVERSITY OF UTAH RESEARCH FOUNDATION; ALVARADO, A., SANCHEZ; REDDIEN) 26 May 2005 (2005-05-26)<br>the whole document<br>-----        | 1-68,<br>70-76        |
| A         | WO 00/01846 A (DEVGEN N.V; PLAETINCK, GEERT; PLATTEEUW, CHRIST; MORTIER, KATHERINE; B) 13 January 2000 (2000-01-13)<br>cited in the application<br>----- |                       |

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/IB2006/002360

## Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

see additional sheet

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Invention 1.: claims 1-68, 70-76 (all partially)

### Remark on Protest

- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: -

Present claims 1-68,70-76 relate to products which have a given desired characteristic, namely being orthologues from insects of the sequences as defined in claims 1 and 2. However, the description does not provide support and disclosure in the sense of Article 6 and 5 PCT for any of such products and there is no common general knowledge of this kind available to the person skilled in the art. This non-compliance with the substantive provisions is to such an extent, that a meaningful search of the whole claimed subject-matter of the claim could not be carried out (PCT Guidelines 9.19 and 9.20).

The search of said claims was consequently restricted to the specifically disclosed products, i.e. those from *Blattella germanica*.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Invention 1.: claims 1-68,70-76 (all partially)

A nucleic acid from *B. germanica* characterised by SEQ ID 1 or its corresponding protein (SEQ ID 2); a double-stranded RNA comprising a strand complementary to SEQ ID 1; constructs and cells comprising said nucleic acid molecules and their use in the manufacture of pesticidal compositions and kits.

---

Inventions 2. to 4.: claims 1-68,70-76 (all partially)

As for subject 1., but concerning respectively the nucleic acids characterised by SEQ IDs 11, 21 and 31, and their corresponding proteins (SEQ IDs 12, 22 and 32).

---

Inventions 5. to 7.: claims 2-22,24-72,75,76 (all partially)

A double-stranded RNA comprising a strand complementary to SEQ IDs 41, 49 or 57 respectively; constructs and cells comprising said nucleic acid molecules and their use in the manufacture of pesticidal compositions and kits.

---

## INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2006/002360

| Patent document cited in search report |    | Publication date |    | Patent family member(s) |  | Publication date |
|--|----|------------------|----|-------------------------|--|------------------|
| WO 0171042                             | A2 | 27-09-2001       | AU | 4594501 A               |  | 03-10-2001       |
| WO 2004049807                          | A  | 17-06-2004       | AU | 2003285594 A1           |  | 23-06-2004       |
|  |    |                  | BR | 0316970 A               |  | 25-10-2005       |
|  |    |                  | CA | 2508422 A1              |  | 17-06-2004       |
|  |    |                  | CN | 1719976 A               |  | 11-01-2006       |
|  |    |                  | EP | 1575363 A1              |  | 21-09-2005       |
|  |    |                  | JP | 2006509009 T            |  | 16-03-2006       |
|  |    |                  | US | 2006051388 A1           |  | 09-03-2006       |
| WO 0246432                             | A  | 13-06-2002       | AU | 1914002 A               |  | 18-06-2002       |
|  |    |                  | EP | 1210875 A1              |  | 05-06-2002       |
| WO 2005047300                          | A  | 26-05-2005       | CA | 2545182 A1              |  | 26-05-2005       |
|  |    |                  | GB | 2422607 A               |  | 02-08-2006       |
| WO 0001846                             | A  | 13-01-2000       | AU | 769223 B2               |  | 22-01-2004       |
|  |    |                  | AU | 4907999 A               |  | 24-01-2000       |
|  |    |                  | BR | 9911802 A               |  | 22-01-2002       |
|  |    |                  | CA | 2332619 A1              |  | 13-01-2000       |
|  |    |                  | CN | 1323354 A               |  | 21-11-2001       |
|  |    |                  | CN | 1657620 A               |  | 24-08-2005       |
|  |    |                  | DE | 1093526 T1              |  | 11-10-2001       |
|  |    |                  | EP | 1093526 A2              |  | 25-04-2001       |
|  |    |                  | GB | 2349885 A               |  | 15-11-2000       |
|  |    |                  | HK | 1029142 A1              |  | 25-04-2003       |
|  |    |                  | HU | 0103571 A2              |  | 28-01-2002       |
|  |    |                  | IS | 5802 A                  |  | 02-01-2001       |
|  |    |                  | JP | 2002519072 T            |  | 02-07-2002       |
|  |    |                  | KR | 20060071438 A           |  | 26-06-2006       |
|  |    |                  | MX | PA00012955 A            |  | 10-04-2002       |
|  |    |                  | NO | 20010019 A              |  | 05-03-2001       |
|  |    |                  | NZ | 509182 A                |  | 30-01-2004       |
|  |    |                  | PL | 347978 A1               |  | 06-05-2002       |

## SEQUENCE LISTING

<110> Commonwealth Scientific and Industrial Research Organisation

<120> Delivery of dsRNA

<130> 500689

<150> AU PR 6215

<151> 2001-07-06

<160> 6

<170> PatentIn version 3.1

<210> 1

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 1

gaattcatgg tccgtcctgt agaaaacc

27

<210> 2

<211> 24

<212> DNA

<213> Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR primer

&lt;400&gt; 2

gaattcccc accgaggctg tagc

24

&lt;210&gt; 3

&lt;211&gt; 27

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR primer

&lt;400&gt; 3

gaattctaga atccaaaaac aaactgg

27

&lt;210&gt; 4

&lt;211&gt; 33

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR primer

&lt;400&gt; 4

ggatcctgac cgtccatcg aataaaatga gcc

33

&lt;210&gt; 5

&lt;211&gt; 23

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR primer

<400> 5  
ccgaaaatcc aatctacgga ccc

23

<210> 6

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 6  
cgacgaataa cctgggctgt tgc

23